Symposia Biologica Hungarica 26

MEMBRANE DYNAMICS AND TRANSPORT OF NORMAL AND TUMOR CELLS

Akadémiai Kiadó, Budapest

MEMBRANE DYNAMICS AND TRANSPORT OF NORMAL AND TUMOR CELLS

26

MEMBRANE DYNAMICS AND TRANSPORT OF NORMAL AND TUMOR CELLS

International Symposium
6–10 July, 1983
Debrecen, Hungary

Edited by

L. TRÓN, S. DAMJANOVICH,
A. FONYÓ and J. SOMOGYI

(Symposia Biologica Hungarica 26)

The cytoplasmic membrane is regarded as a mechanical barrier isolating the structural elements of the cell from the environment. At the same time, it is a well structured matrix allowing the exchange of ions, metabolites (and information) between the interior of the cells and the extracellular space.

Transport phenomena and membrane dynamics constitute the special field of the International Symposium, held in Debrecen, Hungary, in July 1983. Both subjects are targets of intensive scientific research. The latest results and the expected new achievements of these investigations will give a substantial addition to the understanding of the molecular mechanisms of phenomena occurring inside the cells and at the cellular surface. The common symposium of experts, involved in the research of membrane transport and membrane dynamics, facilitated discussions to reveal the interdependence between transport and dynamic features of the structural elements of cells. The majority of the lectures form the basis of this publication; each presentation is followed by a discussion.

This volume will be of interest to biochemists, cell biologists, immunologists, biophysicists and all those working in the field of membrane transport and dynamics.

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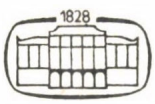

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PREFACE

Membranes are the best known borderlines between different extra- and intracellular compartments in biological entities like cells. The cellular membranes are rather diverse, i.e., most of the cells - among them the eukaryotic ones - have a great number of different types of membranes.

Although it is a commonplace that membranes contain lipid bilayers, the average biologist is possibly less aware of the fact that almost every membrane contains as much proteins as lipids, the latter forming of course the principal diffusion barrier for hydrophilic solutes. In biology one cannot make differences between important and less important components or elements. Now we know that lipids, proteins and polysaccharides are extremely important components of many biological membranes and the priority in importance always depends on the function we are looking for. The outer cytoplasmic membrane separates the extracellular and intracellular spaces approximately at an electric field strength of 10^5 Volt/cm. At the same time many functioning elements can be found inside the membrane regulating the ion transport across the membrane or triggering and/or processing signals coming either from outside or inside the cells. The longstanding Danielli-Dawson model, depicting the membranes or biomolecular lipid layer covered with proteins was substituted by the Singer-Nicolson fluid mosaic membrane model in 1972. The experiments, founding the factual background of the model have introduced a new element into the membrane research, namely dynamics. Membranes are not believed to be rather static structures of the cells any more.

They have structurally related constituents, however, the extent and quality of their interaction is time-dependent, even if there are not known external or internal factors influencing them. Their dynamic interactions with the cytoskeletal elements and many other known and unknown factors from inside the cell, and the continuous signal and information exchange with the environment even in the case of the so-called resting cells make the membrane a never resting biological medium. The membranes control the ionic and molecular traffic of the external and internal parts of cells, trigger signals for excitable cells or even division of the cell initiated by stimulation agents, actually never entering the cells. Intracellular organelles like mitochondria, surrounded by membranes, have been discovered more than hundred years ago, and are still in the focus of interest of bioenergetics. Nuclear membranes are hard to investigate, however, the most modern physical research methods have started to open even this "secret" barrier of the cell interior.

Physics and chemistry, biophysics and biochemistry have contributed much to our knowledge about this colourful, difficult, and still very challenging target of cell biological research.

The principal aim of this book is to present most of the papers presented at the Meeting entitled "Membrane Dynamics and Transport of Normal and Tumor Cells" held at the Debrecen Medical University School, between 6-10 July, 1983. The interesting feature of the Meeting was that the two principal "subclasses" of membrane researchers have been brought together, and membrane dynamics, i.e. lateral and rotational motions and interactions of membrane elements, as well as the major element of transmembrane signalling the ion transport, were jointly discussed by scientists who are outstanding experts of the respective fields. Membrane transport and dynamics are closely, even if not necessarily structurally, related with each other.

The comparison of certain characteristics of normal cells with those of tumor cells intended to bring closer the frontiers of biological research to the possible medical-biological application and thinking.

The willing cooperation of the participants also made it possible to edit the discussions following the individual papers. It helps understanding as well as orientation among really difficult and much debated problems of modern biology.

The reader will not regret to pay attention to the papers and data included in this volume, brought to a readable form in a reasonable short time by the publishing House of the Hungarian Academy of Sciences.

The Editors

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FUNCTIONAL ASPECTS OF MITOCHONDRIAL MEMBRANES AND COMPARTMENTS

A STUDY OF THE INTERMEMBRANE SPACE OF MITOCHONDRIA, WITH SPECIAL EMPHASIS ON THE METABOLIC ROLE OF ADENYLATE KINASE

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INTRODUCTION

The space between the inner and outer mitochondrial membranes, usually referred to as the intermembrane space, contains a number of enzymes (1). One of the best known of these is adenylate kinase, catalyzing the reaction

$$AMP + ATP \rightleftharpoons 2\ ADP$$

There are two types of isoenzymes of adenylate kinase: a "muscle type" of enzyme, which is in the cytosol and whose main physiological function is to convert ADP into ATP and AMP, thus making ADP available as an energy source for e.g. muscle contraction; and a "liver type" of enzyme, found in mitochondria, which has a high affinity for AMP and serves primarily to regenerate ADP from AMP originating from ATP-dependent reactions, making it available for oxidative phosphorylation (see ref. 2 for review). Mitochondrial adenylate kinase thus plays an important role in adenine nucleotide conversions between energy-generating and energy-utilizing processes and thereby the regulation of what has been termed as the "energy charge" or "adenylate charge" of the cell (3). As such the enzyme is subject to extensive control by the nutritional and hormonal states of the organism (4, 5). Moreover, being located in the intermembrane space, mitochondrial adenylate kinase occupies a strategic location in the cell, communicating, on one side, with the mitochondrial matrix through the ADP, ATP translocator across the mitochondrial inner membrane (6), and, on the other side, with the extramitochondrial space through the "pore protein" permeable to ATP, ADP and AMP (and to other low molecular weight solutes) across the mitochondrial outer membrane (7).

The purpose of the work described in this paper was to investigate various parameters of the mitochondrial adenylate kinase of rat liver that may be of interest for a further elucidation of its interaction with extra- and intramitochondrial adenine nucleotides and, in general, the

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role of the intermembrane space in the metabolic interplay between mitochondria and the cytosol. Some experiments with Zajdela hepatoma cells, carried out in our laboratory by Dr. B.D. Nelson and Mr. Firoz Kabir, to be reported in detail elsewhere (8, 9), are also briefly summarized. In these cells, a significant part of the hexokinase is bound to the mitochondrial outer membrane (10, 11), notably to the "pore protein" (12, 13), and is thought to be responsible for their high aerobic glycolysis (10). The data presented here indicate that, while in the liver cell, mitochondrial adenylate kinase interacts primarily with intramitochondrial ATP and ADP, in hepatoma cells it is in close functional contact with the bound hexokinase and, in general, with the extramitochondrial ATP-ADP pool.

EXPERIMENTS WITH LIVER MITOCHONDRIA

The contents of the intermembrane space can be readily isolated by exposing mitochondria to a brief incubation in a hypotonic phosphate buffer at 0°C and subsequent centrifugation according to Bogucka & Wojtczak (14). This treatment disrupts the outer membrane but leaves the inner membrane and the matrix relatively intact. As illustrated by the data in Table I, the intermembrane-space fraction so obtained contains the bulk of the mitochondrial adenylate kinase but is virtually free from marker enzymes of the outer membrane (monoamine oxidase), inner membrane (cytochrome c oxidase) and matrix (malate dehydrogenase). It is also free from low molecular weight components of the matrix, e.g. nicotinamide nucleotides (not shown; cf. ref. 15). On the other hand, it contains 35-40% of the mitochondrial cytochrome c (cf. ref. 15), which probably represents cytochrome c loosely bound to the outer surface of the inner membrane. Whether adenylate kinase also is loosely bound to the outer surface of the inner membrane - or the inner surface of the outer membrane - cannot be decided from the available data. That it would be loosely bound to the outer surface of the outer membrane is made unlikely by the finding that the adenylate kinase activity of intact mitochondria is insensitive to trypsin, while the enzyme solubilized upon disruption of the outer membrane is highly trypsin-sensitive (16).

Table I

Marker-enzyme activities recovered in the isolated intermembrane-space fraction of rat liver mitochondria. Intermembrane-space fraction was isolated according to Bogucka and Wojtczak (14).

Marker enzyme	Percent activity recovered in the intermembrane-space fraction
Adenylate kinase	94
Monoamine oxidase	0
Cytochrome c oxidase	0
Malate dehydrogenase	6
Cytochrome c	35

Table II

Release of mitochondrial Mg^{2+} upon incubation under different conditions.

Rat liver mitochondria were incubated in a medium containing 0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, and 0.5 mg mitochondrial protein/ml for 10 min at 30°C. The samples were centrifuged and the Mg contents of the supernatants were determined by atomic absorption. The Mg content of the mitochondria prior to incubation was appr. 30 nmoles/mg protein.

Additions	Percent of total Mg released
None	0
2 mM EDTA	51.3
2 mM ADP	49.4
2 mM ATP	48.0

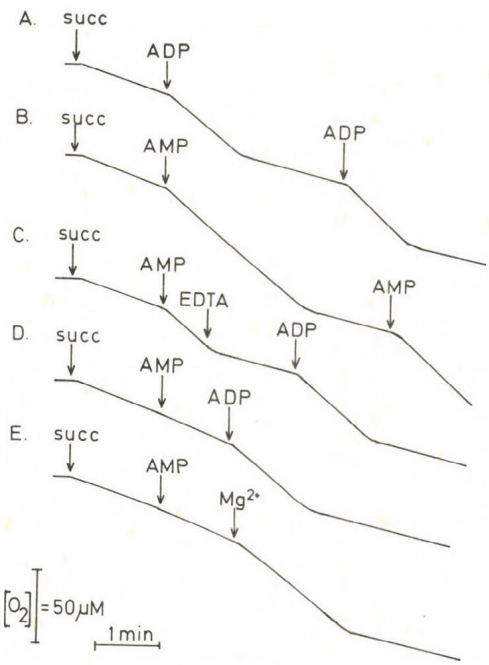

Fig. 1. Oxygen-electrode recordings of rat liver mitochondria respiring under various conditions. The incubation medium consisted of 0.25 M sucrose, 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 2 mM K-phosphate, 3 μM rotenone, and 3 mg mitochondrial protein in a final volume of 2 ml. Temp. 30°C. Additions when indicated were: 5 mM succinate (succ), 170 μM ADP or AMP, 2 mM EDTA and 2 mM Mg^{2+}. In traces d and e, the mitochondria were washed with 2 mM EDTA prior to incubation.

Bogucka & Wojtczak have reported (14, 17) that the intermembrane space contains about one-half of the mitochondrial Mg^{2+}, bound to a protein that is different from adenylate kinase. As shown in Table II, this percentage of the mitochondrial Mg^{2+} can be removed from mitochondria upon incubation with 2 mM EDTA, ADP or ATP, i.e. reagents which bind Mg^{2+}. Prolonged incubation did not remove more Mg^{2+}. EDTA does not penetrate inner membrane; ADP and ATP do, through the ADP,ATP translocator, but this does not translocate the Mg^{2+} chelates of the adenine nucleotides (6). Thus, these data are consistent with the conclusion that the Mg^{2+} removed by EDTA, ADP or ATP represents Mg^{2+} located in the intermembrane space.

Fig. 1 shows that, like ADP, AMP was able to stimulate the respiration of mitochondria incubated in the presence of substrate (succinate) and phosphate (traces A and B); the respiratory rate obtained with AMP was similar to that with ADP, whereas the AMP/O ratio was half the ADP/O ratio, which is consistent with the fact that one molecule of AMP reacts with one molecule of ATP to form two molecules of ADP. The effect of AMP was initiated by EDTA (trace C) and by diadenosine pentaphosphate, an inhibitor of adenylate kinase (18) (not shown), while the effect of ADP was not. Furthermore, the effect of AMP was abolished by washing the mitochondria with EDTA while, again, that of ADP was not (trace D). Upon addition of Mg^{2+} to the EDTA-washed mitochondria, the effect of AMP was restored (trace E). These findings are consistent with the conclusion that EDTA selectively removes Mg^{2+} from the intermembrane space. The translocation of ADP across the mitochondrial inner membrane does not require Mg^{2+} (6), but ATP synthesis does, indicating that the EDTA treatment did not affect the mitochondrial matrix. These results show that the mitochondrial adenylate kinase can utilize, and is dependent on, Mg^{2+} present in the intermembrane space.

Mitochondrial adenylate kinase is known to react with externally added ATP and AMP in an atractylate-insensitive manner (19). This reaction can be followed by using a catalytic amount of ATP and an ATP-regenerating system such as pyruvate kinase and phospho-enol-pyruvate. The reaction can be initiated by the addition of AMP and followed by coupling it to the lactate dehydrogenase system and measuring the oxidation of NADH spectrophotometrically (Fig. 2A). This reaction can also be initiated in the absence of added ATP, but it then occurs with a lag period (Fig. 2B), which can be shortened by preincubating the mitochondria for 10 min. at 30°C prior to the addition of AMP (Fig. 2C). In accordance with earlier observations by Klingenberg et al. (20), this reaction is insensitive to carboxyatractylate, indicating that the ATP does not originate from the mitochondrial matrix. These results show that the intermembrane space contains some ATP and ADP, which can initiate adenylate kinase reaction in the presence of added AMP. Preincubation prior to the addition of AMP allows conversion of ADP to ATP and thus accelerates the attainment of maximal steady-state rate upon the addition of AMP. Direct estimates of the ADP and ATP contents of the isolated intermembrane-space fraction by enzymic analysis showed that about 10% of the total mitochondrial adenine nucleotide was present in this compartment.

Table III compares the kinetic parameters K_m and V_{max} of the adenylate kinase reaction and oxidative phosphorylation as determined in intact mitochondria. The K_m values of adenylate kinase for AMP, ADP and ATP were similar to those reported for the purfied enzyme (21); and the K_m of oxi-

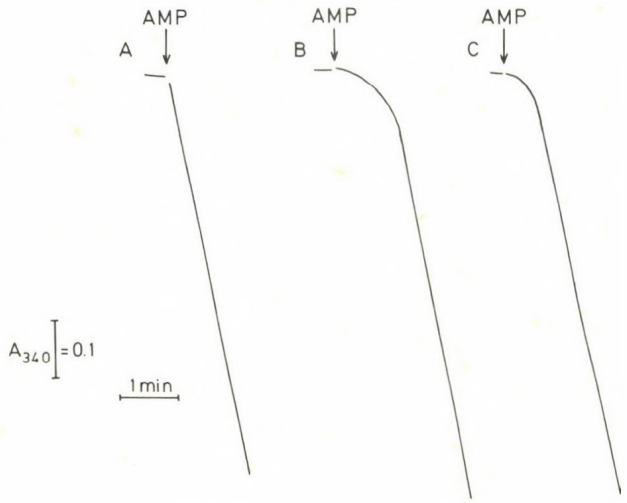

Fig. 2. AMP-induced ATP formation via rat-liver mitochondrial adenylate kinase. The incubation medium consisted of 0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, 133 mM KCl, 1.1 mM Mg^{2+}, 3 μM rotenone, 5 μg oligomycin, 0.2 mM NADH, 2 mM phospho-enol-pyruvate, 5 U pyruvate kinase, 5 U lactate dehydrogenase and 0.4 mg mitochondrial protein in a final volume of 3 ml. NADH oxidation was followed at 340 nm. Temp. 30°C. Additions when indicated were 0.4 mM AMP and 0.7 mM ATP. In trace c, the mitochondria were preincubated for 10 min. at 30°C prior to the addition of AMP.

dative phosphorylation for ADP agreed well with earlier data in the literature (cf. e.g. 22). It may be seen that oxidative phosphorylation has a considerably higher affinity for ADP than has adenylate kinase. On the other hand, the maximal velocity of adenylate kinase in either direction is about 10 times higher than that of oxidative phosphorylation. It should be pointed out, however, that the latter value may vary with the respiratory substrate used (in the present case succinate) and may not be a reflection of the actual V_{max} of either the ATP synthase or the ADP, ATP translocator.

Table III

Kinetic parameters of adenylate kinase and oxidative phosphorylation in rat liver mitochondria.

Reaction	Substrate	K_m (μM)	V_{max} (nmol·min^{-1}mg^{-1}prot)
Adenylate kinase	AMP	50	} 1100
	ATP	75	
	ADP	180	900
Oxidative phosphorylation	ADP	28	91

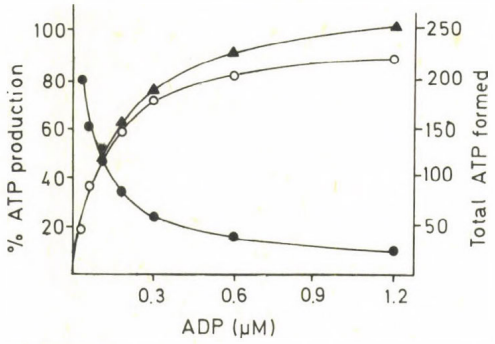

Fig. 3. Relative contributions of adenylate kinase and oxidative phosphorylation to ATP formation by rat liver mitochondria in the presence of varying concentrations of ADP. The rate of ATP formation was determined in a reaction mixture containing 0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, 133 mM KCl, 1.1 mM $MgSO_4$, 2 mM succinate, 2 mM phosphate, 3 μM rotenone, 0.4 mM $NADP^+$, 1 mM glucose, 5 U hexokinase, 5 U glucose-6-phosphate dehydrogenase and 0.5 mg mitochondrial protein, in a final volume of 1 ml. The relative contributions of adenylate kinase (o-o) and oxidative phosphorylation (•-•) were determined by including 5 μg carboxyatractylate and 0.5 mM diadenosine pentaphosphate, respectively, in the reaction mixture. Temp. 30°C. Total ATP formed is expressed in nmoles/min/mg protein.

Fig. 3 is a comparison of the rates of ATP production by adenylate kinase and oxidative phosphorylation, measured in intact mitochondria with succinate as substrate, in the presence of an excess of glucose and yeast hexokinase and of varying concentrations of ADP. The rate of glucose-6-phosphate formation was measured by following the reduction of $NADP^+$ spectrophotometrically in the presence of glucose-6-phosphate dehydrogenase. The results show that, as would be expected from the kinetic data in Table III, at low concentrations of ADP more ATP is formed by oxidative phosphorylation than by the adenylate kinase reaction, while the converse is true at high ADP concentrations.

Fig. 4 compares the rates of glucose-6-phosphate formation at varying concentrations of ADP and AMP. In contrast to the case when no hexokinase and gluxose were present, where ADP and AMP gave the same maximal rates of ATP formation (cf. Fig. 1), in the present case the maximal rate of oxidative phosphorylation (measured here as glucose-6-phospahte formation) was considerably lower with AMP than with ADP. In addition, the rate of glucose-6-phosphate formation showed a marked lag period with AMP, especially at low AMP concentrations (not shown). These results indicate that part of the ATP formed under these conditions is used for the adenylate kinase reaction and only part is available for hexokinase reaction.

The results described above are consistent with the notion (Fig. 5) that the adenylate kinase of liver mitochondria serves to convert AMP formed by cytosolic ATP-dependent reactions - such as fatty acid or amino acid activation - into ADP. The ATP required for this reaction is mainly generated by oxidative phosphorylation taking place in the inner mitochondrial compartment and is furnished to the intermembrane space though the ADP, ATP translocator. The ADP so formed re-enters the matrix by the

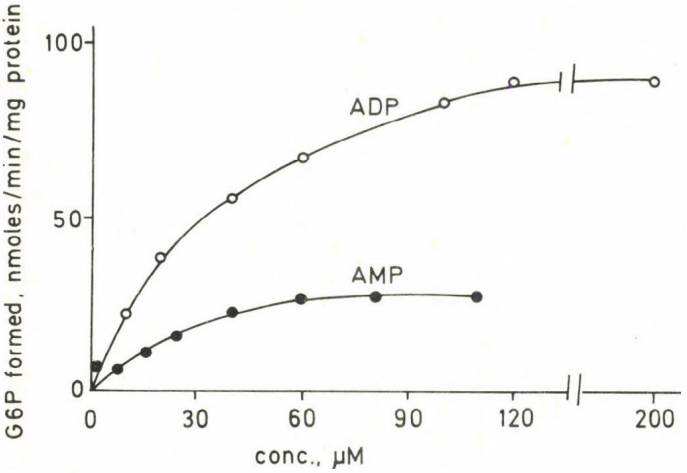

Fig. 4. Oxidative phosphorylation by rat-liver mitochondria in the presence of hexokinase and glucose and of varying concentrations of ADP or AMP. Conditions as in Fig. 3.

same route and is utilized for oxidative phosphorylation. The intermembrane space has its own pool of Mg^{2+} which is not bound to, but can be utilized by, the adenyalte kinase; a fraction of bound ATP and ADP, present in the intermembrane space, may be instrumental in this process.

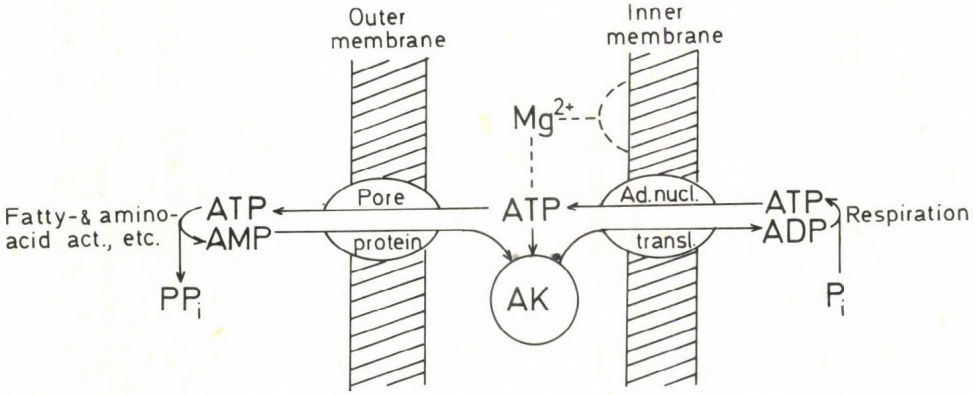

Fig. 5. Schematic view of the functional relationship of adenylate kinase to extra- and intramitochondrial adenine nucleotides in rat liver mitochondria.

EXPERIMENTS WITH HEPATOMA MITOCHONDRIA

A distinctive feature of hepatoma mitochondria, in comparison with mitochondria from liver and several other tissues, is their high content of bound hexokinase. That mitochondria can bind hexokinase, which thereby is activated towards intramitochondrial ATP, was first noticed in the ear-

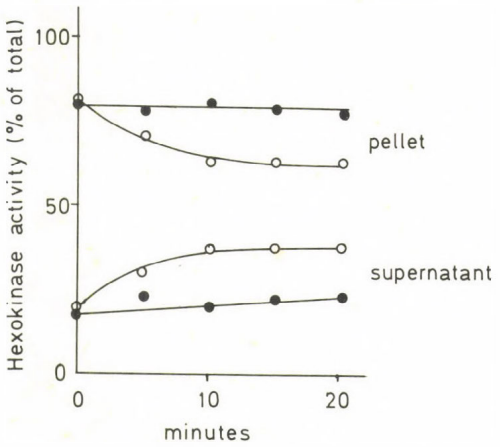

Fig. 6. Effect of glucose on the intracellular distribution of hexokinase in Zajdela hepatoma cells. Cells were suspended to 10 mg protein/ml in a modified Schiebers medium (65 mM KCl, 71 mM NaCl, 6 mM Na-phosphate, pH 7.4, and 3.5 mM $MgSO_4$) buffered to pH 7.4 with 20 mM HEPES, either in the presence or absence of 20 mM glucose. The samples were incubated at 30°C, and at the indicated times 200 µl (2 mg protein) were removed and added to an Ependorff tube containing digitonin to give a final concentration of 40 µg/mg protein. Samples were incubated for 30 sec. and centrifuged for 30 sec. in an Ependorff centrifuge. The samples were then processed and the fractions assayed for hexokinase activity. The mean values are shown for two separate experiments. Hexokinase activity (100%) varied from 130 to 160 nmoles/min/mg whole cell protein for the different time points. (From ref. 8).

ly 1950s with isolated heart (23) and liver (24, 25) mitochondria and yeast hexokinase. In 1953 Crane and Sols (26) reported that brain mitochondria contain a significant part of the tissue hexokinase - a finding that was subsequently confirmed and extended by several laboratories (27-31). In 1967 Rose and Warms (27) found a similar distribution of hexokinase in tumor cells and showed that the outer membrane of the mitochondria in these cells contains a binding site for hexokinase. This binding site has recently been identified with the "pore protein" by Lindén et al. (12) in our laboratory and independently by Fiek et al. (13). The binding of hexokinase to tumor mitochondria has been extensively studied in several laboratories (28-34) and it has been suggested that the mitochondrially bound hexokinase may be instrumental in the high aerobic glycolysis of tumor cells.

Based upon the observations that hexokinase can be released from isolated brain (29, 30) and tumor (27, 29-33, 35) mitochondria *in vitro*, and that bound and soluble hexokinase exhibit different kinetic characteristics (29-31, 35), it was proposed that metabolite-induced release of hexokinase could regulate glycolysis *in vivo* (34;see 29-31 for review). This possibility was recently investigated in our laboratory (8) using Zajdela hepatoma cells grown in the ascites form in Sprague-Dawley rats. Cell sus-

Table IV

Effect of inhibitors on the activity of bound hexokinase of Zajdela hepatoma mitochondria in the presence of added ADP and glucose.

(From Nelson and Kabir (9)).

Hepatoma mitochondria (145 μg protein) in 1 ml buffer containing 50 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 10 mM succinate, 5 mM K-P_i, 1.5 μM rotenone, 1 mM $NADP^+$, 1 mM glucose, 1 U glucose 6-phosphate dehydrogenase, and when indicated, 500 μM ADP or ATP, 3 μg oligomycin, 5 μg carboxyatractylate (CAT), 1 μM ClCCP, and 200 μM diadenosine pentaphosphate (AP_5A).

Exp No.	Additions	Hexokinase activity (μmoles·min^{-1}·mg^{-1} protein)
1	ADP	92
	ADP + oligomycin	100
	ADP + ClCCP	105
	ADP + oligomycin + ClCCP	100
	ADP + oligomycin + ClCCP + CAT	95
	ATP	222
2	ADP	38.5
	ADP + CAT	41.5
	ADP + AP_5A	0
3	ADP	191
	ADP + oligomycin	191
	ADP + oligomycin + CAT	191

pensions in a modified Schriebers medium (see legend of Fig. 6) were incubated with glucose and at suitable intervals samples were removed and subjected to rapid fractionation by digitonin to separate the cytosol from the mitochondria (36); the concentration of digitonin was so chosen as not to affect the mitochondrial outer membrane (for details, see ref. 8). The addition of glucose caused, as expected, an instantaneous decrease in respiration due to active glycolysis (Crabtree effect). As shown in Fig. 6, however, there occurred only a moderate decrease in the extent of mitochondrially bound hexokinase, from about 80 to about 65%, and this change was relatively slow, hardly compatible with a physiological regulatory mechanism.

As another possibility to explain the functional significance of mitochondrially bound hexokinase it appeared interesting to investigate the relationship of the bound hexokinase to the mitochondrial adenylate kinase. For this purpose isolated Zajdela hepatoma mitochondria were incubated with substrate (succinate), phosphate, ADP and glucose, and the amount of glucose-6-phosphate was measured by supplementing the system with glucose-6-phosphate dehydrogenase and $NADP^+$ and following the formation of NADPH spectrophotometrically. The results of three experiments are shown in Table IV. It may be seen that the amount of glucose-6-phosphate formed was unaffected by inhibitors of oxidative phosphorylation such as oligomycin or the uncoupler ClCCP, or by carboxyatractylate, an inhibitor of the ADP, ATP translocator. On the other hand, glucose-6-phosphate for-

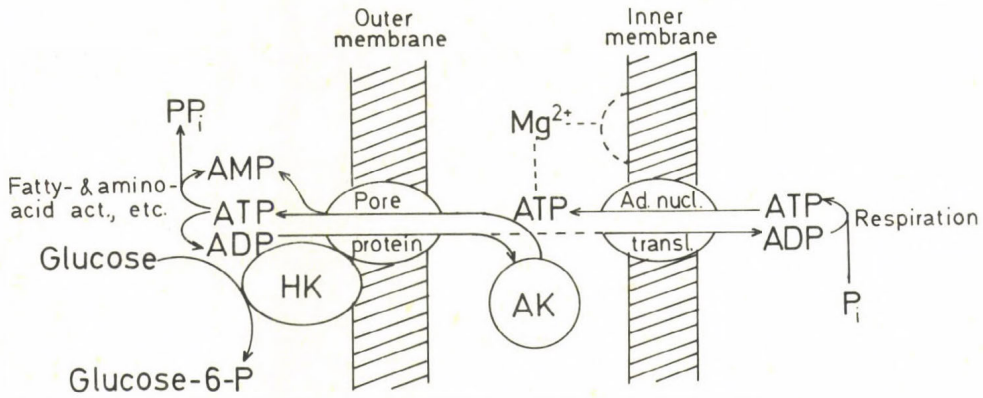

Fig. 7. Schematic view of the functional relationship of adenylate kinase to extra- and intramitochondrial adenine nucleotides in hepatoma mitochondria.

mation was complementary inhibited by diadenosine pentaphosphate, an inhibitor of adenylate kinase. In other experiments, the latter inhibition was less complete, but the bulk of the glucose-6-phosphate formation could invariably be accounted for as proceeding by way of adenylate kinase. The variation in the amounts of glucose-6-phosphate formed in the three experiments in Table IV reflects a variation of the amount of bound hexokinase in different hepatoma-cell preparations. The amount of glucose-6-phosphate formed in the presence of added ATP (Table IV, expt. 1) was about twice that in the presence of added ADP, indicating that the adenylate kinase reaction was rate-limiting. The ready reactivity of the mitochondrially bound hexokinase with externally added ATP seems to be in contrast with the situation found in liver, where the small amount of hexokinase bound to mitochondria has been reported to be relatively inaccessible to extramitochondrial ATP (37).

Although these findings are still preliminary and in need of further experiments, it is conceivable that the mitochondrial adenylate kinase of hepatoma cells, similar to the liver enzyme, has its main function to regenerate ADP from AMP formed by cytosolic ATP-dependent reactions. However, while the liver enzyme carries out this function mainly in conjunction with the oxidative phosphorylation system of the inner membrane-matrix compartment, the adenylate kinase of hepatoma mitochondria interacts primarily with the bound hexokinase and, in general, with the glycolytic system of the cytosol (Fig. 7). That this close contact between the mitochondrial adenylate kinase and bound hexokinase may involve the "pore protein" of the outer membrane, which is the binding-site of hexokinase (11, 12), is an intriguing possibility that deserves further exploration.

ACKNOWLEDGEMENT

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DISCUSSION

BHARGAVA:

Is anything known about what happens in regenerating liver: is there an increased association of the hexokinase with the outer membrane? I wonder if this may be a general property of all dividing cells and not confined to just tumor cells.

ERNSTER:

Binding of hexokinase to mitochondria seems to be most extensive in tissues with a high rate of glycolysis. I am not aware of any information concerning regenerating liver.

SCHONER:

Looking for the regulation of carbohydrate metabolism in the regenerating liver one has to look for this phenomenon with other means than the usual homogenate biochemistry. At least we found in a histochemical study using antibodies against pyruvate kinase isoenzymes that in regenerating liver pyruvate kinase type M_2 was found only in the regenerating wound tissue and not in the intact tissue.

WOJTCZAK:

What is your evidence that a large portion of cytochrome c is present in the intermembrane compartment? If cytochrome c is present there, one should expect a high rate of the oxidation of external NADH by intact mitochondria. And this is not the case, at least for liver mitochondria.

ERNSTER:

You are right. Probably the cytochrome c recovered in the intermembrane-space fraction is in the intact mitochondrion loosely bound to the outer surface of the inner membrane.

SCHONER:

Can one say that the glycolysis is compartimentalized when one finds 2 types of hexokinases, a membrane and a free from with 2 different kinetic properties?

ERNSTER:

Yes, especially as there is a special hexokinase-binding protein on mitochondria (now identified as the pore protein).

SCHINDLER:

Hexokinase has C-terminal binding domain for pore protein interaction.

Effect of cytoskeletal interactions on mitochondrial shape and activity?

ERNSTER:

Indeed, the involvement of the cytoskeleton in determining the shape and movement of mitochondria and the interaction of mitochondria with the rest of the cell is a striking possibility that was amply emphasized by Gottfried Schatz in his plenary lecture at the Strasbourg meeting to which I was referring at the end of my talk.

SOMOGYI:

Are there any data available whether 5'-AMP deaminase is localized together with adenylate kinase in liver mitochondria?

ERNSTER:

To my knowledge AMP deaminase is a cytosolic enzyme, not associated with mitochondria.

LIGETI:

The pore protein of the outer membrane was found to have a molecular weight of 30 000. All the transport proteins isolated from mitochondria so far have a molecular weight

around 30 000. Can it be a mere coincidence or might it have some more significance?

ERNSTER:

This is an interesting question, the answer to which will have to await knowledge of the primary structures of the various transport proteins.

Symposia Biologica Hungarica 26 (1984)

CALCIUM ION DEPENDENT TRANSMITTER RELEASE IN A DIFFERENTIATING GANGLION CELL-DERIVED HUMAN NEUROBLASTOMA CELL LINE SHSY-5Y

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INTRODUCTION

The induction of differentiation and development of an excitable membrane has extensively been studied in various murine neuroblastoma cell lines (Kimhi et al., 1976). In the presence of various inducers these cells grow long neurite-like processes and ion channels like those of excitable cells appear in their plasma membrane. Pahlman et al. (1981) have found that the treatment of a human ganglion cell-derived neuroblastoma cell line SHSY-5Y with the phorbol ester 12-o-tetradecanoyl-phorbol-13-acetate (TPA) induces the growth of long neuritelike processes from the cells indicative of neural differentiation. We have shown (Akerman et al., 1983) that concomitant with morphological differentiation the various functional properties of excitable cells are expressed as well. Thus the electrical potential across the plasma membrane of the differentiating cells increases from -40 mV to -60 mV. Furthermore a depolarization-induced-verapamil-sensitive Ca^{2+} influx can be demonstrated.

As a response to electrical excitation in nerve cells Ca^{2+} channels are opened allowing Ca^{2+} ions to flow into the cells (Åkerman and Nicholls,1983). The resulting increase in cytosolic Ca^{2+} ion activity is the trigger of transmitter liberation from nerve endings.The aim of the present study was to investigate maturation of these mechanisms involved in neural transmission in the human neuroblastoma cell line SHSY-5Y.

EXPERIMENTAL

Cells of the neuroblastoma clone SHSY-5Y (kindly donated by Dr K.Nilsson,Uppsala,Sweden) were grown as monolayer cultures in petri dishes in the presence of 10^{-7}M TPA in RPMI 1640 medium for 3 days. In order to induce ^{3}H-noradrenaline and $^{86}Rb^{+}$ uptake the culture medium was aspirated off and substituted with a medium containing: 122 mM NaCl,5mM KCl,1.2 mM $MgCl_2$,1 mM $CaCl_2$,0.44 mM KH_2PO_4,4.2 mM $NaHCO_3$,5mM D-glucose, 1mM ascorbate,1 mM nialamide,20 mM TES pH 7.4 together with 0.25 µM ^{3}H-noradrenaline and 0.3-3 µM ^{86}RbCl. After incubation for 1 hr at 37°C the monolayers were washed four times with the Na^{+} based experimental medium to remove external isotope. Thereafter the washout of isotopes was continued,using 0.5 ml of the Na^{+} based medium with 1 min intervals. 10 µM imipramine was included in the washout medium to prevent the function of the noradrenaline uptake mechanism during washout. The washouts were collected for counting. High K^{+} depolarization was obtained by isosmotically replacing Na^{+} with K^{+}.

RESULTS

Under conditions described in the methods section, the cells take up 18 ± 2 % of the added ^{3}H-noradrenaline. The uptake is reduced to 4 ± 1 % in the presence of 10 µM imipramine,an inhibitor of noradrenaline uptake by noradrenergic nerve endings.

Fig. 1 shows typical washouts of ^{3}H-noradrenaline and $^{86}Rb^{+}$ (as a marker of K^{+} release or plasma membrane depolarization).When the cells are depolarized by increasing external K^{+} to 50 mM a fast and transient release of the prelabelled ^{3}H-noradrenaline occurs and there is a sustained release of $^{86}Rb^{+}$. A similar release of noradrenaline is observed in the presence of the Ca^{2+} ionophore A23187 (1 µM). In contrast to the effect of high K^{+} this compound does not cause any significant increase in $^{86}Rb^{+}$ release,suggesting that the

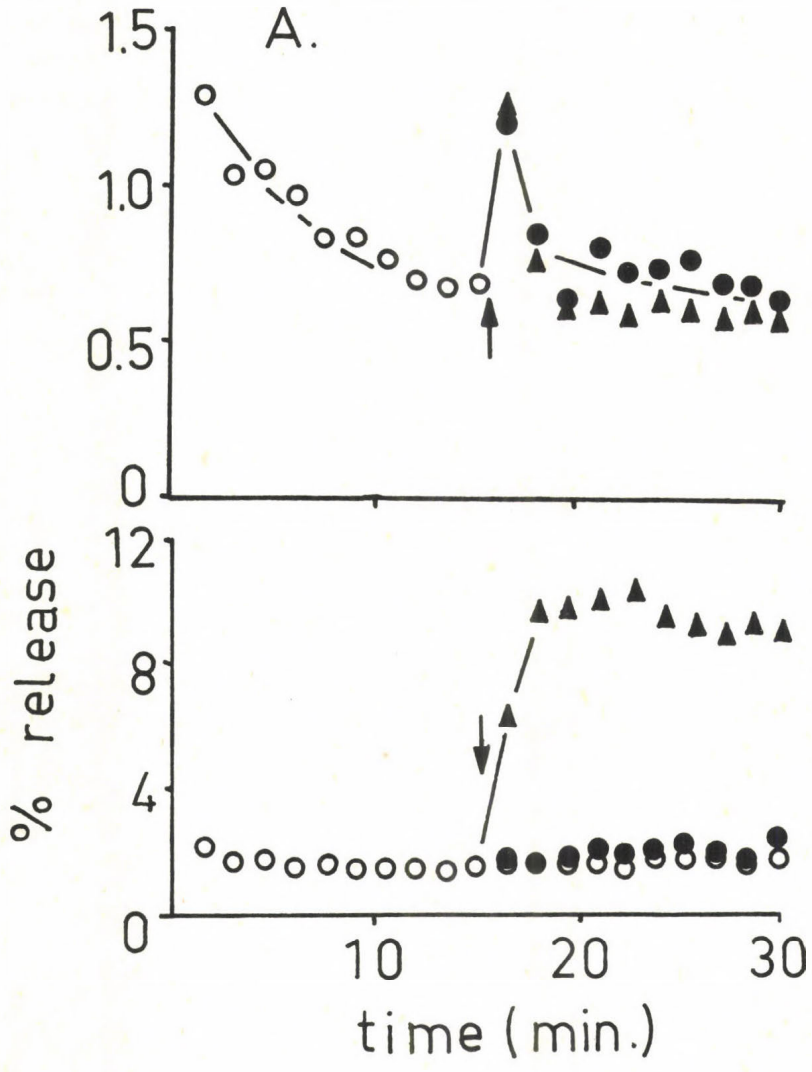

Fig.1. Washouts of ^{3}H-noradrenaline and $^{86}Rb^{+}$ from prelabelled neuroblastoma cell monolayers.A. ^{3}H-noradrenaline,B. $^{86}Rb^{+}$. Addition at arrow: 50 mM KCl (▲) or A23187 (●).Results expressed as percental loss from the amount in monolayer immediately prior to each wash.

effect of the ionophore is simply a result of an increase in cytosolic Ca^{2+} rather than being due to depolarization. The effect of high K^+, but not that of ionophore A23187, is inhibited by the Ca^{2+} channel blocker, verapamil (not shown).

Since the preganglionic transmitter in sympathetic ganglia is acetylcholine the effect of this substance on ^{3}H-noradrenaline release was tested. Fig. 2 shows that acetylcholine causes a small but reproducible release of ^{86}Rb and ^{3}H-noradrenaline.

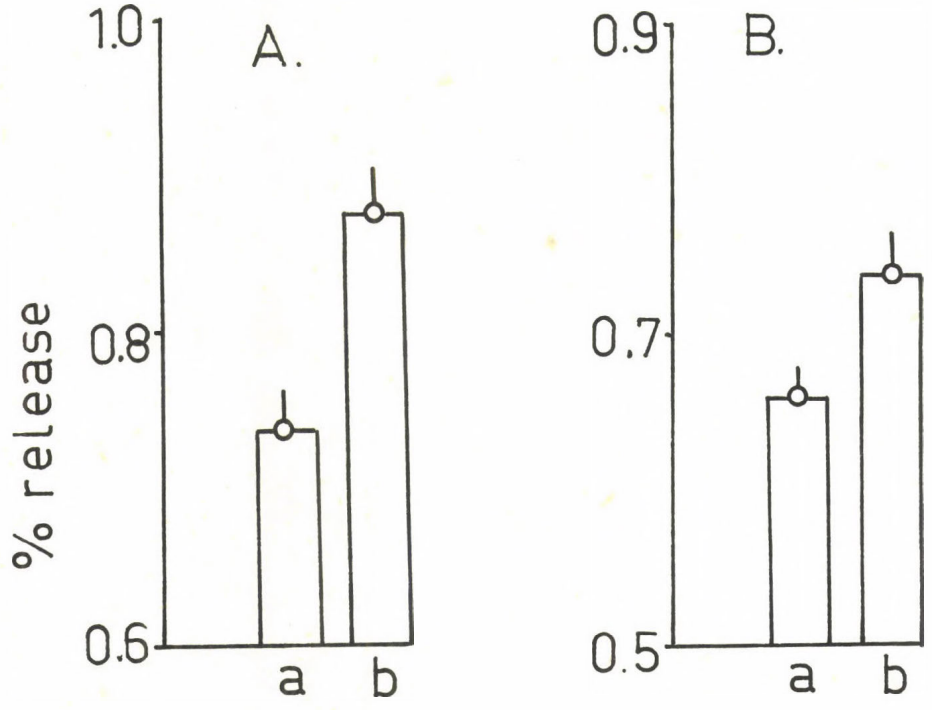

Fig.2. Release of ^{86}Rb and ^{3}H-noradrenaline by acetylcholine. Conditions as in Fig.1.A. $^{86}Rb^{+}$, B. ^{3}H-noradrenaline. Control (a) or in the presence of 100 µM acetylcholine. (b) ±SEM, N=6

0.3 mM ouabain was present in this experiment in order to prevent the repolarization after acetylcholine induced depolarization and thus amplify the response.

DISCUSSION

The results of the present study show that as a response to TPA treatment of the human neuroblastoma cell line SHSY-5Y there is a considerable maturation towards ganglion cells. Thus an imipramine sensitive mechanism for the uptake of noradrenaline, the endogenous transmitter in ganglion cells, as well as depolarization induced release of preloaded noradrenaline are observed.

A Ca^{2+} dependent exocytotic mechanism for the liberation of the transmitter is indicated since a similar release is obtained by the application of Ca^{2+} ionophore A23187. We have previously shown (Åkerman et al, 1983) that depolarization causes an increase in Ca^{2+} uptake of these cells. This increase in Ca^{2+} uptake as well as transmitter liberation are inhibited by the Ca^{2+} channel antagonist verapamil, suggesting that the opening of Ca^{2+} channels triggers transmitter release as in the case with isolated nerve endings (Åkerman and Nicholls, 1983).

The release of noradrenaline induced by acetylcholine, the natural agonist for the activation of ganglion cells, is of considerable interest. The smaller response in this case as compared to high K^+ and A23187, is probably due to a much smaller and more transient depolarization induced by the agonist. It is also possible that only part of the cells expresses acetylcholine receptors.

SUMMARY AND CONCLUSIONS

The in vitro differentiation of the human neuroblastoma cell line SHSY-5Y leads to expression of several typical functional properties of mature ganglion cells including excitability, Ca^{2+} channels and transmitter (noradrenaline) release. Thus this cell line offers a model for studies on pharmacological and biochemical mechanisms, involved in noradrenergic transmission, and also for studies on cell differentiation.

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DISCUSSION

KOVÁCS:

I wish to know what was the concentration of veratridine and verapamil used by you?

My second question is: what kind of evidence have you got for the location of acetylcholine-receptor on the presynaptic membrane?

AKERMAN:

Usually 100 μM veratridine and 75 μM verapamil. In titration experiments we find depolarization with as low as 1 μM veratridine and a maximal change around 5 μM.

We think that the acetylcholine receptor localizes to the cell body. We have no information on presynaptic receptors.

LIGETI:

In your experiments measuring intracellular calcium concentration upon addition of veratridine, gramicidin, etc., did you have Ca^{2+} in the external medium?

How did you express Rb^{+} release?

Did you measure intracellular Ca-concentration changes also upon K^{+}-depolarization?

AKERMAN:

Yes.

We express ^{86}Rb release as a percent release compared to the previous sample.

Yes, but we have not titrated it with synaptosomes but with 10 mM K^{+} we have seen an increase in intracellular Ca^{2+}.

FONYÓ:

What is the role of mitochondria of the cell line in the secondary Ca^{2+} sequestration (after the Ca^{2+} influx phase) and in the Ca^{2+} release phenomenon can this Ca^{2+} come eventually from the mitochondria?

AKERMAN:

We have not looked into the problem yet. However, with synaptosomes we have shown that a main part of depolarization induced Ca^{2+} uptake in synaptosomes is localized to the mitochondria.

SOMOGYI:

Vizi and coworkers have found several years ago, that the inhibition of (Na^{+}/K^{+})-ATPase by ouabain, Ca, SH-reagents, etc., cause a significant increase of neurotransmitter release from brain slices. They stress the primary role of the temporary blocking of Na-pump in the release mechanism of neurotransmitters. Could you comment on this hypothesis?

AKERMAN:

In synaptosomes we see a depolarization with ouabain but not in this cell culture at the time period measured, about 8 min. This is probably due to different surface area volume relationship. I have not read the paper by Vizi et al., and have no explanation for the discrepancy.

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CONTROL OF THE MOLECULAR ACTIVITY AND THE SUBSTRATE AFFINITY IN MONOMERIC AND DIMERIC BOVINE CYTOCHROME C OXIDASE

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Cytochrome c oxidase (EC 1.9.3.1) is the terminal component of the mitochondrial electron transport chain (Keilin 1966). It is composed of several polypeptides ranging from 2 to 13 depending on the organism (Ludwig 1980, Gennis et al. 1982, Schatz & Mason 1974, Werner 1977. The bovine heart enzyme, for which the largest structural (Fuller et al. 1979) and kinetic information is available, contains 13 polypeptides (Merle et al. 1982) part of them can be considered bona fide subunits, is still however a controversial matter (Azzi 1980, Capaldi et al. 1982)
Cytochrome c oxidase has four prosthetic groups , two hemes a and two coppers, which are located most probably within the two largest subunits (Winter et al. 1980, Corbley & Azzi 1983). The physiological electron donor of cytochrome c oxidase is reduced cytochrome c which binds with the area surrounding the exposed heme edge to the second largest subunit of cytochrome c oxidase (Bisson et al. 1978, Bisson et al. 1980). Another site of interaction, although not directly implied in electron transfer, occurs between the region near the C-terminus of cytochrome c and the third largest subunit of cytochrome c oxidase (Birchmeier et al. 1978, Fuller et al. 1981). Kinetically, two interaction sites have been described for cytochrome c, one of high affinity ($K_{d=10^{-8}}$M) and a second with lower affinity ($K_{d=10^{-6}}$ M) (Ferguson-Miller et al. 1976, Ferguson-Miller et al. 1978).

Cytochrome c oxidase has been shown to be active as a dimer (Robinson & Capaldi 1977, Saraste et al. 1981), although indications of the existence of active monomers have been underevaluated (Darley-Usmar et al. 1982). In the dimer a cleft exists delimited by the second largest subunit of one monomer and the third of the other; this would be the physical site of cytochrome c binding with high affinity (Capaldi et al. 1982). The kinetically defined low affinity site has been related to the amount of cardiolipin of the cytochrome c oxidase (Vik et al. 1981).

A second reaction of cytochrome c oxidase, namely the vectorial proton translocation coupled to the oxidation reaction process of the enzyme has been identified (Wikström 1977), and the third largest subunit has been indicated to be involved in such a process (Casey et al. 1980). The concept of two physically distinct sites both catalytically competent in the electron transfer process, originally proposed by the group of Margoliash (Ferguson-Miller et al. 1976, Ferguson-Miller et al. 1978), has been extended to the H^+ translocation reaction which is apparently restricted according to Wikström to the so-called high affinity site (Wikström & Penttilä 1982).

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More recently Fuller et al. (1981) proposed that the low affinity site is not catalytically competent but it is responsible for the activation of the high affinity site.

In this paper the kinetics of cytochrome c oxidation by bovine heart cytochrome c oxidase have been re-analysed in experimental conditions, in which the enzyme is largely monomeric, dimeric or in larger aggregates. The experiments presented in this study are consistent with a model in which only one cytochrome c binding site per monomer of cytochrome c oxidase exists. In the dimer of the enzyme the two high affinity sites are physically at a close distance, so that once the first site is occupied the second molecule of cytochrome c has steric and charge induced difficulties to occupy its site. Such a situation results in an apparent negative cooperativity or, by simplification in two apparent binding sites having different affinities. The present data suggest that dimerization of the enzyme plays a key role in determining the off-rate constant of the reaction between cytochrome c and cytochrome c oxidase. Since this constant is the rate limiting step in the steady state kinetics of the enzyme (Nicholls 1974) it appears that the dimerization is also controlling the V_{max} of cytochrome c oxidase.

MATERIALS AND METHODS

Materials. Cytochrome c from horse heart, type VI was from Sigma. Dodecylmaltoside was from Calbiochem or synthesized according to Rosevear et al. (1980), cholate from Fluka, Triton X-100 and Tween-80 from Merck. Dextran blue, and Sephadex G-100 were from Pharmacia, Ultrogel AcA 34 was from LKB. Catalase was from Boehringer, ferritin and aldolase from Serva. Immunoglobulin G was a gift from Dr F. Hesford.

Preparation of cytochrome c oxidase . Cytochrome c oxidase was prepared according to Yu et al. (1975).

Determination of the relative hydrodynamic radii. The apparent M_r was estimated by determining the protein hydrodynamic radius relative to a set of standards (ferritin, catalase, aldolase and IgG) using an Ultrogel AcA 34 column (1x45 cm) equilibrated with the detergent dodecylmaltoside (0.1%) in 10 mM Tris-HCl, pH 7.4 (Rosevear et al. 1980) in the presence or absence of 50 mM KCl.

Miscellaneous techniques. The protein concentration was estimated by the biuret method (Gornall et al. 1949). Polyacrylamide gel electrophoresis was carried out according to Kadenbach et al. (1983) or Swank & Munkres (1971) using bovine serum albumin, creatin kinase, carboanhydrase, myoglobin and cytochrome c as M_r standards. Gels were scanned in a special attachment of the Aminco DW-2a spectrophotometer at 570-530 nm after staining with Coomassie blue. The activity of cytochrome c oxidase was assayed polarographically according to Rosevear et al. (1980) and spectrophotometrically (Ferguson-Miller et al. 1978) in an Aminco DW-2a spectrophotometer at 550-540 nm using the extinction coefficients 19.4 $mM^{-1}.cm^{-1}$ and 24 $mM^{-1}.cm^{-1}$, reduced minus oxidized for cytochrome c and cytochrome c oxidase, respectively. Integrations, curve-fitting and simulations were carried out in Commodore 2001 and 8032 computers equipped with a BBC-Servogor analog plotter.

RESULTS

The spectrophotometric determination of the activity of cytochrome c oxidase in the presence of the non-ionic detergent Tween-80 and at a concentration of 50 mM KCl as a function of cytochrome c concentration is shown in Fig. 1.

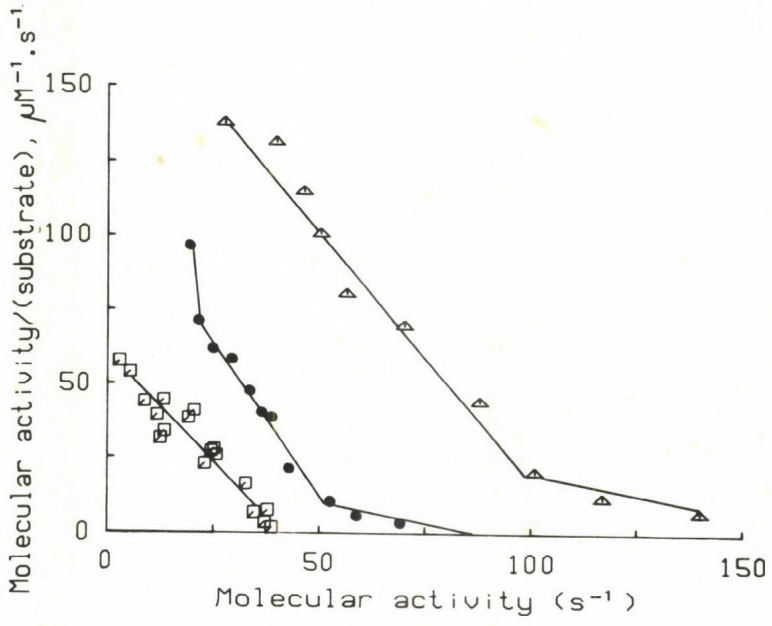

Figure 1. Eadie-Hofstee plots of the activity of native cytochrome c oxidase at high and low ionic strength. The activity was measured spectrophotometrically as described in the Methods in 10 mM Tris-HCl, pH 7.4. The concentration of reduced cytochrome c was 0.2-20 uM and the detergent present either dodecylmaltoside or Tween-80. ( ) no KCl, 0.1 % dodecylmaltoside; ( ) 50 mM KCl, 0.1% dodecylmaltoside; ( ) 50 mM KCl, 0.1% Tween-80. The concentration of cytochrome c oxidase was 0.6 nM and the molecular activity is given in nmol cytochrome c oxidized/s / nmol heme a.

The data plotted according to the graphic representation of Eadie-Hofstee indicate the existence of at least three kinetic phases or of a continous variation of the apparent affinity of the enzyme for reduced cytochrome c and of its V_{max}.

When the detergent employed for the activation and dispersion of the enzyme was dodecylmaltoside also in the presence of 50 mM KCl the kinetic picture was similar, although in this case only two major kinetic components in the Eadie-Hofstee plot could be observed. A completely different result was obtained using the same detergent dodecylmaltoside, but in the absence of added KCl, the only ionic strength derived in this case from the buffer present (10 mM Tris-HCl pH 7.4). In these experimental conditions only one phase of high affinity and low V_{max} was observed.

From this experiment it can be inferred that the multiplicity of the kinetic phases in the reaction of cytochrome c with cytochrome c oxidase is more than an intrinsic property of the enzyme a consequence of the type of detergent and ionic strength present in the system. The data plotted according to the Hill equation were fitted with a straight line of slope 0.57, 0.53, 1.01 for the Tween-80, the dodecylmaltoside in the presence of KCl and the dodecylmaltoside in the absence of KCl, respectively (Fig. 2). In all experiments regression coefficients were higher than 0.97.

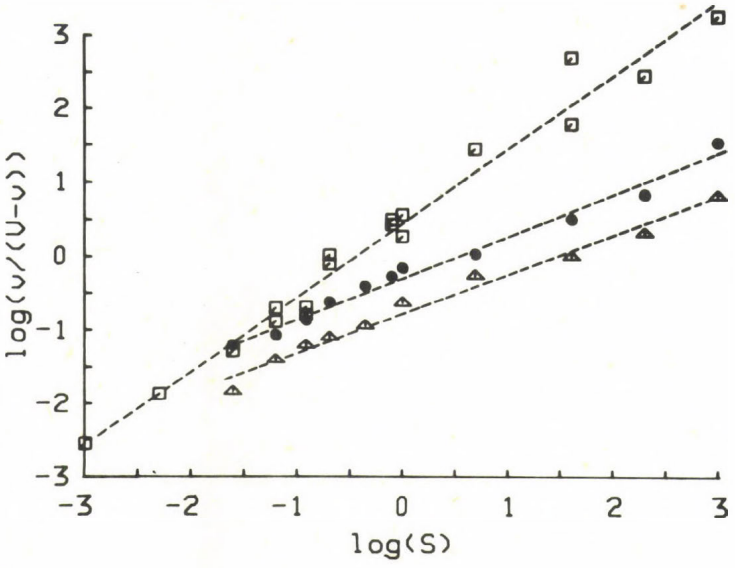

Figure 2. Hill plots of the activity of native cytochrome c oxidase at high and low salt concentration. Data and symbols are as in Fig. 1.

The experiments of Fig. 1 and 2 imply that at high salt an apparent negative homotropic cooperativity exists consistent with the hypothesis that binding of a second molecule of cytochrome c to the enzyme decreases the affinity but increases the V_{max} of cytochrome c oxidase. At low salt the cooperativity disappears; the enzyme has lower molecular activity and cytochrome c has high affinity for the cytochrome c oxidase.

TABLE I

Apparent molecular weights of cytochrome c oxidase forms. Measurements were made by filtration on Ultrogel AcA 34 (see Methods). The values in parentheses refer to the relative amounts of the molecular species.

Conditions	Molecular species, M_r	
150 mM KCl	340,000 (100%)	---
50 mM KCl	340,000 (100%)	---
10 mM KCl	340,000 (80%)	110,000 (20%)
0 mM KCl	aggregates(20%)	160,000 (80%)

Table I shows the data of the gel filtration of native cytochrome c oxidase through Ultrogel AcA 34 after previous equilibration with 10 mM Tris-HCl pH 7.4, 0.1% dodecylmaltoside and 50 mM KCl. A single peak with an apparent M_r of 340,000 was eluted. The subunit analysis of this enzyme according to Kadenbach et al. (1980) indicated the presence of 12-13 polypeptides for a calculated minimum M_r of 170,000 (Verheul et al. 1981). The result is consistent with that previously obtained by Rosevear et al. (1980) and indicates also that a dimer is the species present under the experimental conditions employed. This conclusion is further supported by the similar values obtained in other detergents (in 0.1% Triton X-100 and in 0.1% Tween 80 the M_r values at high salt concentrations were 380,000 and 400,000, respectively), in which the enzyme has been established to be dimeric by independent techniques (Darley-Usmar et al. 1981, Robinson & Capaldi 1977, Saraste et al. 1981, Love et al. 1970). When the gel filtration was carried out in the absence of added KCl more than two thirds of the protein were eluted with a fraction corresponding to an apparent M_r of about 110,000 indicating the presence of a monomeric enzyme (Love et al. 1970), which contained, as indicated by an electrophoretic analysis, all the 12-13 subunits of the native enzyme (Fig. 3)

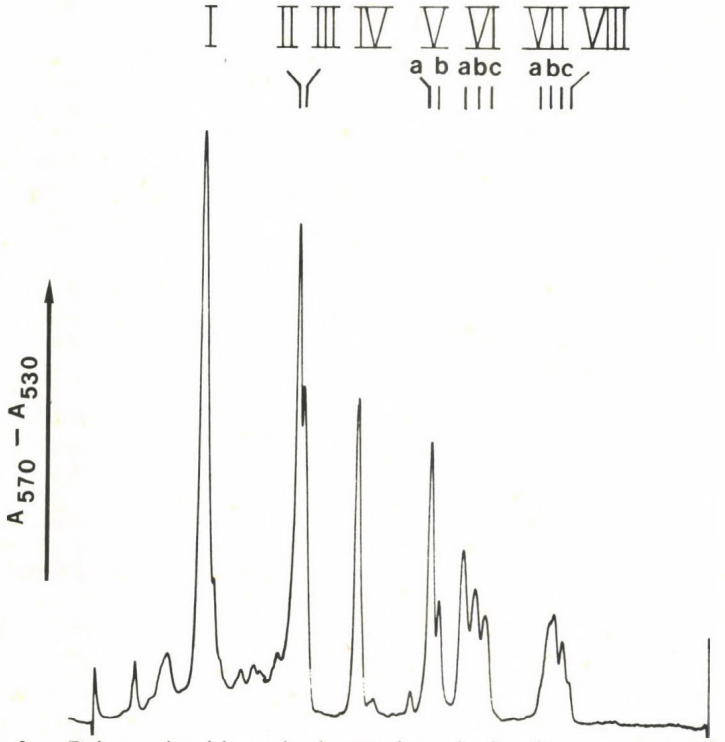

Figure 3. Polyacrylamide gel electrophoresis in the presence of Na^+-dodecyl sulfate of cytochrome c oxidase. The conditions employed can resolve 12-13 polypeptides (Kadenbach et al. 1983). The numbers and letters correspond to the numbering of subunits according to Robinson & Capaldi (1977).

Re-chromatography of the species having 110,000 M_r resulted in a change of this value to 160,000. Considering the anomalous migration of highly asymmetric molecules such as cytochrome c oxidase the results can be interpreted only to indicate that the enzymatically active molecular species containing 12 polypeptides with an apparent M_r of 110,000-160,000 is a monomer.

The possiblity of a retardation effect on the cytochrome c oxidase dimers induced by the low ionic strength, producing only apparently a low molecular weight species was considered. At some intermediate value of salt concentration such a situation should result in a diminished retardation of the protein, i.e. an apparent higher M_r. The result was instead the appearance of a species corresponding to a M_r of 340,000 with a diminution of the one at 110,000, without the appearence of any intermediate species. It seems thus that the low ionic strength condition does not affect the rate of elution of cytochrome c oxidase in the Ultrogel AcA 34 column, but it rather controls the monomer-dimer equilibrium of the enzyme.

DISCUSSION

The data obtained using bovine heart cytochrome c oxidase in different experimental conditions, with different detergents, low or high ionic strength, monomer or dimer enzymes, suggest that a two, non-interacting binding site model is not sufficient to explain the steady state kinetic behaviour of this enzyme. Moreover the data of covalent binding of cytochrome c did not suggest the existence of a physically distinct second site, but rather of a half-of-the-sites-reactivity (Bisson et al. 1980). Measurements of the stoichiometry of binding using gel filtration techniques demonstrated the existence of a single binding site under conditions of reactant concentrations which should have allowed demonstration of the putative second site (Ferguson-Miller et al. 1978). Moreover a careful kinetic characterization of the reaction of cytochrome c with cytochrome c oxidase has given no indication of two distinct catalytic sites (Antalis & Palmer 1982). Finally even the finding of the existence of two distinct binding sites for cytochrome c on cytochrome c oxidase would not necessarily imply that they are also involved in the catalytic function of the enzyme. The novelty of this study consists in the use of a recently adopted detergent (Rosevear et al. 1980) to disperse the enzyme homogeneously and to maximally activate it. Under these optimal conditions the molecular species of cytochrome c oxidase and their kinetic behaviour were investigated. It was found that the enzyme, normally a dimer, under very low ionic strength conditions became monomeric. The kinetics of the two species were different, the dimers having a biphasic saturation behaviour, the monomers a monophasic one. This phenomenon was found to be fully reversible (not shown). After chromatography in Ultrogel AcA 34 at low salt concentration, the monomer of cytochrome c oxidase had monophasic kinetics in low salt (Hill coefficient = 1.0) and polyphasic kinetics (Hill coefficient less than 1) in 50mM KCl. In order to account for the experimental data reported above and for those already present in the literature the following model is presented. (Fig. 4).

Cytochrome c oxidase when in a dimeric state possesses two cytochrome c binding sites, namely one per monomer. As indicated by Capaldi's group, the cleft created by the two adjacent monomers constitutes the site of cytochrome c binding (Capaldi et al. 1982). In each molecule of cytochrome c the area of the exposed heme edge, containing lysyl 13 residue, interacts with the second largest subunit of a cytochrome c oxidase monomer near the histidyl 161 residue indicated to be within the stretch of aminoacids of this subunit in close contact with the area of cytochrome c enclosing lysyl 13 residue (Bisson et al. 1982).

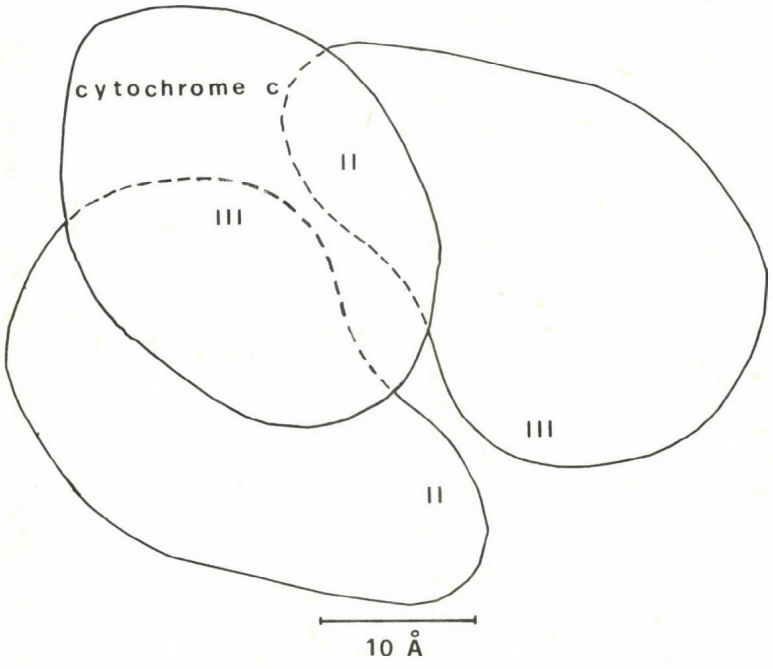

Figure 4. Schematic model of a cytochrome c oxidase dimer interacting with cytochrome c. The model represents a top view of the two monomers of cytochrome c oxidase redrawn from Fuller et al (1979). The Roman numbers refer to the relative subunit and its position. Only one molecule of cytochrome c is drawn. It appears difficult to insert the second molecule without overlapping with the first. Steric hindrance as well as charge repulsion between cytochrome c molecules may be taken as a physical basis for the apparent negative cooperativity discussed in the text.

The other side of the cytochrome c molecule interacts with the third largest subunit of the other monomer. Such an interaction creates the conditions for increasing the off-rate constant of cytochrome c with consequent increase in the V_{max}.

Non-cooperative enzyme kinetics may occur each time when the enzyme is monomeric or in experimental conditions when the choice of the species of cytochrome c and cytochrome c oxidase which react is such that the interaction between the third largest subunit and cytochrome c is weaker. This may be the situation for the yeast cytochrome c and the bovine cytochrome c oxidase (cf. Fig. 7 in Ferguson-Miller et al. (1976). It may also be the case which follows extraction of cardiolipin from the enzyme, resulting in an oxidase with low activity and high affinity for cytochrome c. The estimation of the molecular dimension of the cleft between two monomers of cytochrome c oxidase in which cytochrome c binding occurs indicates that the space available to two cytochrome c molecules cannot exceed approximately 4-5 nm, the transverse diameter of the complex. It is evident that due to the molecular dimension of cytochrome c (2-3 nm) the occupation of one binding site by one molecule will sterically hinder the occupation of the second site by the second molecule in the dimer. This situation can lead to an apparent negative cooperativity up to the half-of-the-sites reactivity which has also been described for the binding of cytochrome c to cytochrome c oxidase (Bisson et al. 1980).

In the case of cytochrome c oxidase dimers the interaction between two molecules of the substrate, cytochrome c, may not be hindered only by the sterical properties of the adjacent sites but also by the effect of the strong electrical dipole of a cytochrome c molecule on the other one (Koppenol & Margoliash 1982). An apparent negative homotropic cooperativity in the cytochrome c oxidase kinetic behaviour is obtained not only with the spectroscopic assay used in this study, but also with a polarographic assay and after replotting a large number of the data published in the literature obtained by using both assays (cf.Ferguson-Miller et al. 1976, Ferguson-Miller et al. 1978, Darley-Usmar et al. 1981, Rosevear et al. 1980).

Different forms of cytochrome c oxidase have been described to exist, "resting" and "pulsed" (Brunori & Wilson 1982). It would be of interest to investigate whether a correlation exists between these two forms and the two extreme situations of structure and reactivity described in this study. It has been shown that the type of detergent, the ionic strength and possible other situations still under investigation are responsible for the transition between monomers and dimers of cytochrome c oxidase. An extrapolation may be made on this basis, namely that the different kinetic behaviours of cytochrome c oxidase obtained in the presence of different types of phospholipids may be due to different proportions of aggregates, dimers and monomers of the enzyme under those conditions. Also in vivo the activity of cytochrome c oxidase may be regulated by the same parameters.

ACKNOWLEDGEMENTS

We like to thank Dr. Ganka Kossekova for the very stimulating discussions during the initial part of this work. The financial support of the Schweizerischer Nationalfonds (N.3.739-0.80) of the Sandoz Stiftung and of the Emil-Barrel Stiftung is gratefully acknowledged.

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ERNSTER:

Obligatory coupling of electron transfer to H^+ translocation - as documented in experiments with e.g. "cytochrome oxidase vesicles" - should result in the inhibition of electron transfer if H^+-translocation is inhibited. How do you explain that the inhibition of H^+ translocation through subunit III by DCCD did not cause an inhibition of electron transfer?

AZZI:

The results of our laboratory indicate that the inhibition of the H^+-pump is associated with a progressive inhibition of electron transport. The inhibition of the electron transport reaction is however always less than that of H^+ translocation. No increase in proton permeability is observable as a consequence of DCCD binding to the enzyme. The situation can be operationally defined as "molecular uncoupling".

WOJTCZAK:

The difficulty in interpreting kinetic data for enzymes like cytochrome oxidase is that, under in vivo conditions, neither the enzyme nor the substrate are free in solution. My question is therefore, how the results you showed, coming from studies on soluble cytochrome oxidase, can be related to the situation in mitochondria. My second question is a technical one. In your last slide showing the effect of ionic composition on kinetic properties of cytochrome oxidase in intact mitochondria, how was the permeability barrier of the inter membrane from cytochrome c overcome.

AZZI:

It has been shown that the kinetics of cytochrome c oxidation in the solubilized enzyme and in the membrane bound

one are analogous (Fergusson-Miller et al., 1976, 1978). One should bear in mind that the solubilized enzyme is inserted in a micelle of detergent which simulates the membrane situation. As to the kinetics of cytochrome c oxidation in mitochondria, the preparation employed was frozen and thawed, with subsequent fragmentation of the outer membrane, and removal of the permeability barrier for cytochrome c.

DAMJANOVICH:

A part of my question has been asked by Dr. Wojtczak. However let me make some comments on the salt dependent aggregation and dissociation of the cytochrome oxydase. The "salting in" and "salting out" effects are well known for solubilized enzymes. It is interesting that principally the same effects can be found with enzymes bound to membranes.

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SOME NOVEL ASPECTS CONCERNING THE MOLECULAR MECHANISM OF CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM

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INTRODUCTION

Considerable information is available concerning various methods of induction of Ca^{2+} release. Release of Ca^{2+} from SR in vitro can be induced by several different methods: e.g. a) an increase of the extravesicular [Ca^{2+}] to the order of several uM (Ca^{2+}-induced Ca^{2+} release, refs. 8,9,14,19,25,27,28), b) the addition of drugs such as caffeine (7,8,17,19,24,27,28) and quercetin (19,20) alone or with Ca^{2+}, c) substitution of permeable anions for impermeable ones or substitution of impermeable cations for permeable ones (depolarization-induced Ca^{2+} release, refs. 3,5,8,15,16,19,26), d) the addition of proton ionophores (31), organic anions (21,32), and e) chemical modification of -SH groups (1).

However, many of the studies of in vitro Ca^{2+} release reported in the literature should be re-investigated in terms of physiological significance. If the experimentally produced Ca^{2+} releases described above are to be candidates for a physiological mechanism, at least two requirements should be met. a) Both the rate and the amount of Ca^{2+} release should be sufficiently large, and b) the Ca^{2+} release should be followed by Ca^{2+} re-uptake to account for the transient tension development and decline in the intact muscle fiber. These requirements have not yet been fully satisfied in the in vitro experiments.

Through the recent studies (19) several clues for experiments that satisfy the above requirements have begun to emerge. One of the key factors is the type of preparation. According to many reports (3,5,19,24,27,28) heavy fractions of SR are enriched in Ca^{2+} release functions relative to light fractions, suggesting that the putative Ca^{2+} channels are located chiefly in the terminal cysterna portion of the SR membrane (19), from which a heavy fraction originates (23). Furthermore, as demonstrated in this article, the transverse tubular (T-tubule) system attached to SR plays a crucial role in triggering rapid Ca^{2+} release. Thus, it appears that an ideal system for studies of rapid Ca^{2+} release in vitro is the purified membranes consisting of the

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T-tubule/SR complex (13). The lack of reversibility in many of the in vitro Ca^{2+} release experiments seems to be ascribable at least partly to the fact that many of those experiments were carried out in the absence of an ATP-regenerating system. As demonstrated in our recent paper (19), the large and slow Ca^{2+} release extensively reported in the literature is the artifact due to the consumption of ATP during the prolonged reaction. In the presence of an ATP-regenerating system, the rapid Ca^{2+} release is followed by a spontaneous re-uptake of Ca^{2+} as demonstrated here. Thus, it appears that the in vitro Ca^{2+} release discussed here is consistent with the physiological mechanism both in terms of the rapidity and the reversibility of Ca^{2+} release.

The main purpose of this article is to summarize the recent advances in our studies of the molecular mechanism of Ca^{2+} release in vitro. Stopped-flow measurements of the rapid kinetics of Ca^{2+} release have permitted distinction and characterization of the receptor and channel functions involved in the triggering and Ca^{2+} efflux mechanisms, respectively. These studies have also brought some insights into the mechanism by which the T-tubule communicates with SR. The results with several inhibitors of Ca^{2+} release may help in designing future experiments by which the key molecular components responsible for Ca^{2+} release may be identified.

RESULTS AND DISCUSSION

A. Kinetic characterization of calcium release induced by various methods of triggering

a. General

Fig. 1 depicts representative time courses of Ca^{2+} release induced by caffeine (A), quercetin (B), and replacement of K^+ with $choline^+$, or membrane depolarization (C), which were monitored by changes in the absorbance of arsenazo III with the use of a stopped-flow multiple channel spectrophotometer system described elsewhere (19). As shown in the traces on a longer time scale (Figs. 1 A_1, B_1, and C_1), in the presence of an ATP-regenerating system, all of these types of Ca^{2+} release (downward excursion) are finished within a few seconds, and followed by a spontaneous re-uptake phase, showing that the release is transient or reversible (see Introduction). The reversibility also indicates that the Ca^{2+} efflux produced in vitro is not due to an irreversible breakdown of the membrane permeability barrier.

In the initial descending phase of the trace (Fig. 1 A_2, B_2, C_2), Ca^{2+} release follows a single exponential decay in the cases of caffeine and quercetin-induced Ca^{2+} release: $A \exp(-kt) + A_o$. In the case of depolarization-induced Ca^{2+} release, however, a two-exponential model had to be used for fitting: $A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_o$. The amount (A) and the rate constant (k) of Ca^{2+} release were calculated from iterative computer fitting using the above equations; the initial rate of Ca^{2+} release was calculated from A and k, since the initial slope of the trace is $-A.k$.

b. Caffeine and quercetin-induced calcium release

The effects of increasing concentrations of caffeine and quercetin were investigated. In the presence of optimum [Ca^{2+}] (e.g. 10 μM), the initial rate of Ca^{2+} release (A.k) is sharply stimulated by increasing concentrations of caffeine and quercetin in the concentration range of 0.5-4.0 mM and 10-200 μM, respectively. The V_{max} value was calculated from 1/V (V=A.k) versus 1/[drug] plots (Table I). It should be noted that the V_{max} values for caffeine and for quercetin are about the same, although the effective drug concentration ranges differ

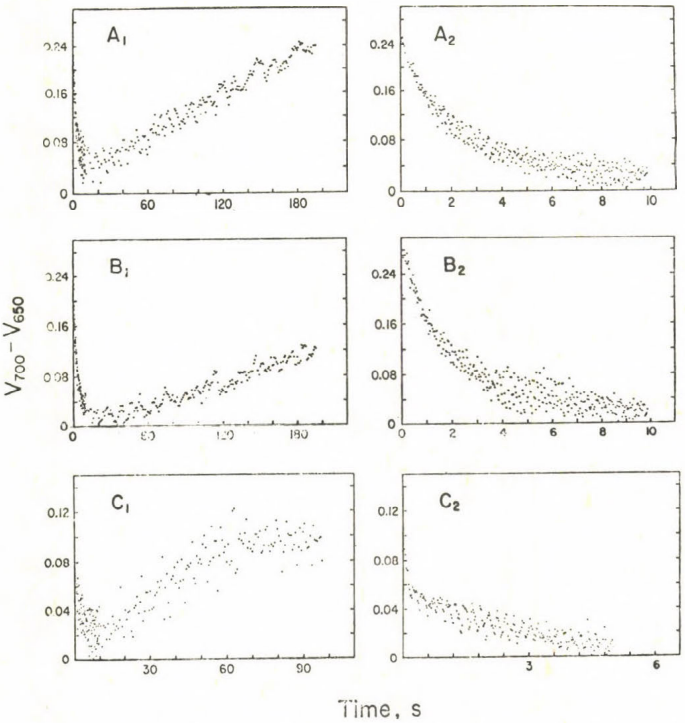


Figure 1. Stopped-flow recording of rapid and reversible Ca^{2+} release induced by caffeine (A), quercetin (B) and ionic replacement (C). Active Ca^{2+} loading was started by adding 0.5 mM Mg.ATP to a solution containing 150 mM KCl, 1.6 mg/ml SR, 20 mM MES (pH 6.8), 50 μM $CaCl_2$, 2.5 mM PEP, 10 units/ml pyruvate kinase and 9 μM arsenazo III, and immediately loaded in syringe A of a Durrum stopped-flow apparatus and incubated at 27°. At the steady state of Ca^{2+} uptake Ca^{2+} release was triggered by mixing one part of the syringe A solution with one part of the syringe B solution containing various triggering agents: 4 mM caffeine (A), 100 μM quercetin (B) and 0.15 M choline Cl (C). To determine the time course of Ca^{2+} release, changes in the transmittance of arsenazo III at 700 and 650 nm were recorded through interference filters built in an air-turbine, converted to optical density with a demodulator, and then recorded in a PDP 11 03 computer. Downward excursion represents release of Ca^{2+} and upward excursion represents the reuptake of the released Ca^{2+}.

TABLE I Kinetic parameters of caffeine and quercetin-induced Ca^{2+} release.

	V_{max} nmol Ca^{2+}/mg/s	K_m
caffeine	21.0	0.2 mM
quercetin	25.2	3.3 μM

considerably. The calculated Hill coefficient is very close to 1 in both cases, suggesting that the number of drug binding sites is one per one receptor unit. In view of the above fact that the V_{max} values are about the same for caffeine and quercetin-induced Ca^{2+} release, we propose that these drugs compete for the same receptor. In further support of this view, the addition of caffeine and quercetin together produces no further increase of V_{max}. Furthermore, the addition of both caffeine and quercetin at submaximal concentrations produces an additive effect on both A and k.

c. Depolarization-induced calcium release

Although several different methods of ionic replacements were used previously to produce membrane depolarization (3,5,8,15,16,19,26), replacement of K^+ with $choline^+$ was found to be most suitable for the studies of depolarization-induced Ca^{2+} release with arsenazo III for several technical reasons as discussed elsewhere (19).

As described above, one of the characteristics of depolarization-induced Ca^{2+} release is that the time course of Ca^{2+} release consists of two distinguishable phases: rapid and slow. Table II depicts an example of the calculation of the amount (A) and the rate constant (k) of Ca^{2+} release in the rapid (A_1 and k_1) and slow (A_2 and k_2) phases. The equivalent parameters of caffeine and quercetin-induced Ca^{2+} release are also shown for comparison. As seen

TABLE II. Various kinetic parameters of depolarization-induced Ca^{2+} release in comparison with caffeine and quercetin-induced Ca^{2+} release.

Triggering method	A_1	k_1	A_2	k_2	A	k
Depolarization	8.4	2.57	15.4	0.24		
caffeine + Ca^{2+}					45.6	0.44
quercetin + Ca^{2+}					55.8	0.46

in the table the rate constant of the rapid phase (k_1) is much larger than that of caffeine and quercetin-induced Ca^{2+} release (k), whereas the rate constant of the slow phase (k_2) is about the same as the latter (k). In contrast, the total amount of Ca^{2+} released in both phases of depolarization induced Ca^{2+} release ($A_1 + A_2$) is much smaller than that of caffeine and quercetin-induced Ca^{2+} releases (A). These facts suggest the hypothesis that the mechanisms for triggering the

rapid and slow phases of Ca^{2+} release and caffeine or quercetin-induced Ca^{2+} release are all different.

d. Regulation of calcium release by calcium

The initial rates of several different types of Ca^{2+} release (A.k) were investigated as a function of the extravesicular Ca^{2+} concentration ($[Ca_o^{2+}]$). Plots of the Ca^{2+} release rate versus pCa_o (Fig. 2) show a bell-shaped curve, indicating that opening and closing of the putative Ca^{2+} release channel are regulated by $[Ca_o^{2+}]$ (19). Interestingly, the pCa_o-dependence remains to be the same in the presence of potentiators such as caffeine (cf. Fig. 2) and quercetin (not shown). The $[Ca_o^{2+}]$-dependence of the initial rate of depolarization-induced Ca^{2+} release (viz. $A_1.k_1$) is different from that of the other types of Ca^{2+} release in that depolarization-induced Ca^{2+} release is maximally activated in the virtual absence of Ca_o^{2+}.

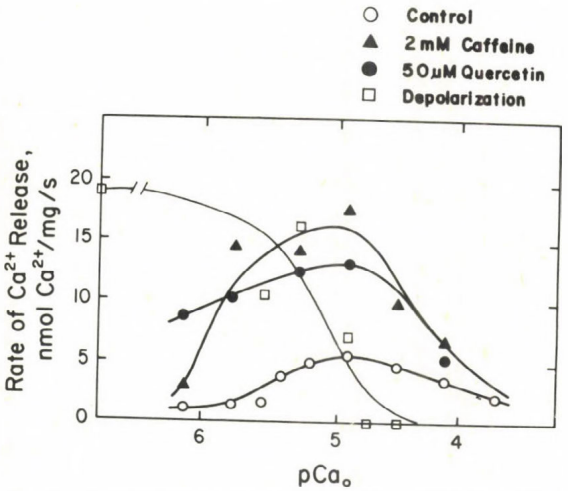


Figure 2. The $[Ca_o^{2+}]$-dependence of the (Ca^{2+} + drug)-induced and depolarization-induced Ca^{2+} releases. Active Ca^{2+} loading and Ca^{2+} release assay were carried out as described in the legend to Fig. 1. The initial rates of Ca^{2+} release were calculated as the derivative at t = 0 of the computer-fitted Ca^{2+} release curves.

The simplest model to account for the above results is as follows. There are at least two different types of receptors (R_1 and R_2) for different triggering signals, which are distinguishable by different $[Ca_o^{2+}]$-dependence of Ca^{2+} release (see Fig. 4). Ca^{2+} binding to R_1 opens Ca^{2+} release channels, which is manifested in the so-called Ca^{2+}-induced Ca^{2+} release. Quercetin and caffeine produce further activation of the Ca^{2+}-induced Ca^{2+} release in such a way that the activation by those drugs is additive to the activation by $[Ca_o^{2+}]$, suggesting that caffeine or quercetin binds to $R_1.Ca$ to produce further activation. The other kind of receptor (R_2) is sensitive to membrane depolarization. Although high affinity Ca^{2+} binding takes place to R_2, this produces an inhibitory effect on Ca^{2+} release. The evidence

discussed in Section B suggests that the receptor class R_2 is resolved further into two subclasses.

We have investigated whether different extents of intravesicular Ca^{2+} loading affect the kinetics of Ca^{2+} release. Briefly, it appears that there are little Ca^{2+} release functions below the critical level of Ca^{2+} loading (approx. 30 nmol Ca^{2+}/mg), suggesting that the filling of such an internal Ca^{2+} compartment is a prerequisite for the activation of the Ca^{2+} release mechanism (D.H. Kim and N. Ikemoto, unpublished results). Above this critical level of Ca^{2+} loading, increasing levels of internal Ca^{2+} loading result in an increase of the amount of Ca^{2+} release (A) in the cases of both (Ca^{2+} + drug)-induced and depolarization-induced Ca^{2+} releases; whereas the rate constant (k) is virtually unaffected by the extents of internal loading.

B. Triggering mechanism of calcium release

a. Role of the T-tubule in depolarization-induced calcium release

In order to investigate the role T-tubule may play in triggering Ca^{2+} release, an SR preparation enriched in the T-tubule/SR complexes was treated with a French press to dissociate T-tubules from SR (4,13); and the French press-treated sample was subsequently incubated in 0.4 M cacodylic acid to re-associate the detached T-tubule (4,13). As described previously, free T-tubule vesicles are located in sucrose density gradient layers lower than 35% and cholesterol is enriched in the T-tubule but virtually absent in SR (29). Therefore, the extent of dissociation and reassociation could be monitored by measuring the cholesterol content of sucrose gradient fractions of the above samples (Table III). Ca^{2+} release was induced by replacement of K^+ with choline$^+$ as was done in the experiments shown in Fig. 1, and the values of A_1, k_1, A_2 and k_2 were calculated by iterative computer fitting. Two examples of such experiments are shown in Table III. As shown here, upon dissociation of the T-tubule, the amount of Ca^{2+} release in the rapid phase (A_1) is considerably decreased. Upon reassociation of T-tubules with SR by incubation with cacodylic acid, the original level of A_1 is almost completely restored. These results clearly indicate that the linkage between the T-tubule and SR is essential for inducing the rapid phase of depolarization-induced Ca^{2+} release.

It is important to note that the slow phase of depolarization-induced Ca^{2+} release is virtually unaffected by dissociation and reassociation of T-tubule (not shown), indicating that T-tubule is not involved in the triggering mechanism of the slow phase. Furthermore, dissociation and reassociation have little effect on the (Ca^{2+}+caffeine)-induced Ca^{2+} release (not shown). Thus, it appears that ionic replacement (depolarization) opens SR Ca^{2+} channels through at least two different routes: one involves the depolarization of the T-tubule; and the other, the depolarization of SR. These results also suggest that the receptors for the slow phase of depolarization-induced Ca^{2+} release and (Ca^{2+}+caffeine)-induced Ca^{2+} release are localized in the SR moiety.

In the light of the above considerations, the receptor R_2 discussed in Section A is further resolved into two subclasses R_{2T} and R_{2SR}

(see Fig. 4). The model assumes that the receptor R_{2T} is sensitive to the changes of the T-tubule membrane potential and is responsible for the

TABLE III. Process of dissociation and reassociation of the T-tubule/SR complex monitored by the fraction of the SR-associated T-tubule. Free and associated forms of the T-tubule in each of three sample types were separated by a discontinous sucrose density gradient. The cholesterol contents of these fractions were determined, and the fraction of SR-associated T-tubule was calculated.

Treatment	Experiment 1	Experiment 2
None	1.00	1.00
French press	0.59	0.64
0.4M cacodylate	1.04	0.85

rapid phase of depolarization-induced Ca^{2+} release; whereas R_{2SR} is sensitive to the potential change of the SR membrane leading to the slow phase of depolarization-induced Ca^{2+} release. The location of R_1, which is responsible for the (Ca^{2+}+drug)-induced Ca^{2+} release (see above), is now assigned to SR.

b. Analysis of the molecular pathway of the triggering route

In view of the postulated mechanism for the Ca^{2+} release in vivo (6,10,22,30) and of the rapidity of Ca^{2+} release triggered via T-tubule in vitro described above, our current efforts are concentrated to resolve various molecular events and the molecular components that are involved in the communication from the T-tubule to SR.

We have found that low concentrations of the Ca^{2+} antagonist nitrendipine (e.g. 10 nM) produce selective inhibition of the rapid phase of depolarization-induced Ca^{2+} release. Nitrendipine primarily affects the A_1 value, which is similar to the mode of inhibition produced by T-tubule detachment (see above).

According to recent reports (11) the binding site of dihydroxypyridines such as nimodipine and nitrendipine is preferentially located in the purified T-tubule membrane (n = 50 pmol/mg T-tubule protein, K_m= 2 nM). We have essentially confirmed this. It is interesting to note that the maximal capacity of binding as well as the extent of inhibition of Ca^{2+} release (10--70% reduction of A_1 with 10 nM nitrendipine) depend on the conditions of assay and on preparations. Furthermore, treatment of T-tubules with detergent increases the binding capacity. These facts suggest that the binding components (presumably T-tubule Ca^{2+} channels) are located in a domain of the T-tubule membrane which is not readily accessible to the reaction solution.

From the above considerations, we propose that Ca^{2+} channels located in the T-tubule membrane may be a part of the molecular pathway of the communication from the T-tubule to SR. A voltage-dependent opening of the T-tubule channel probably permits Ca^{2+} movement from

T-tubules to some putative compartments, which in turn activates the Ca^{2+} release channels in the SR moiety (cf. refs. 2,10). Such a Ca^{2+}-mediated opening of Ca^{2+} release channels should be distinguished from the Ca^{2+}-induced Ca^{2+} release, since the rate constant of the former is much larger than that of the latter as described in Section A. It is likely that the compartments which are used for the passage of the 'messenger Ca^{2+}' are located in the 'feet' (cf. refs. 12,33).

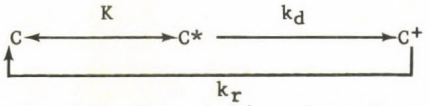
C. Channel functions

a. General

Although several different receptors and routes are distinguishable by the kinetic criteria as described above, some properties described below, which are probably ascribable to the putative Ca^{2+} release channel, are similar regardless of the methods of triggering. 1) The initial rate of Ca^{2+} release (A.k) of the rapid phase of depolarization-induced Ca^{2+} release is about the same as that of (Ca^{2+}+drug)-induced Ca^{2+} release, although the rate constant is much higher than that of the latter. ii) As described in section A, high $[Ca_o^{2+}]$ (>10 μM) inhibits all types of Ca^{2+} release. iii) Channels close spontaneously independent of the method of triggering (see below). iv) Some reagents (e.g. ruthenium red) inhibit all types of Ca^{2+} release (see below). These facts suggest that similar or identical molecular components serve as the Ca^{2+} release channels.

b. Spontaneous closing

Many investigators, who observed non-exponential efflux time courses of the passively loaded $^{45}Ca^{2+}$, have interpreted their data in terms of the existence of several different kinetic classes of the channel (e.g. ref. 25). We have re-investigated this problem by carrying out 'passive' $^{45}Ca^{2+}$ efflux studies, in which there is no Ca^{2+} re-uptake due to the absence of Mg.ATP. As described elsewhere (19), the Ca^{2+} efflux is attenuated spontaneously even in the presence of potentiators such as caffeine and quercetin; and the Ca^{2+} efflux phase has an approximately constant life-time, regardless of the amount and the initial rate of Ca^{2+} release. In order to account for the non-exponential time course, therefore, we have proposed the following model:



The model assumes that the putative channel can exist in three states; C is a ground state that is closed but capable of opening; C*, opened; and C^+, refractory state. It is assumed that the $C \longrightarrow C^*$ conversion is rapid with an equilibrium constant K which is affected by various effectors (e.g. caffeine); whereas the $C^* \longrightarrow C^+$ reaction (viz. spontaneous closing) proceeds slowly with the rate constant k_d, which is apparently unaffected by those effectors. The C^+ state is eventually converted to the C state with the rate constant k_r, allowing the channels to re-open.

Fig. 3 illustrates our recent experiment that further supports the above model. In this experiment, 2 mM caffeine was added to induce release of the actively loaded Ca^{2+} and the time course of Ca^{2+} movement was monitored for a long period of time. As shown here, caffeine produces Ca^{2+} release ($C \longrightarrow C^*$), which is followed by re-uptake phase reflecting the fact that opened channels are spontaneously closed ($C^* \longrightarrow C^+$). After a certain lag phase, which represents the $C^+ \longrightarrow C$ reaction, Ca^{2+} release resumes, since the added caffeine is still available in the reaction solution.

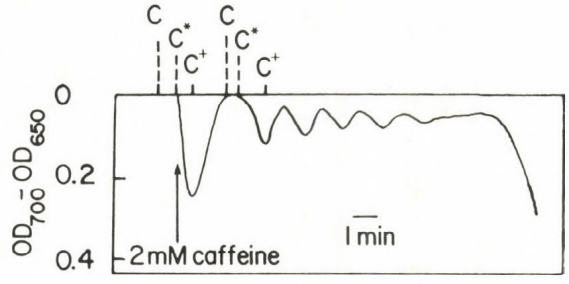


Figure 3. Spontaneous cycling of Ca^{2+} release and uptake after the addition of caffeine. Active Ca^{2+} loading was done as described in the legend to Fig. 1. After the steady state was reached, as determined by $A_{700}-A_{650}$ of arsenazo III with the use of a Perkin-Elmer spectrophotometer (model 557), 2 mM caffeine was added to initiate Ca^{2+} release and the subsequent changes in $A_{700}-A_{650}$ of arsenazo III were recorded.

The oscillatory behavior could not be observed in the case of depolarization-induced Ca^{2+} release presumably due to the fact that the membrane potential change is transient. However, the observation that released Ca^{2+} is rapidly re-accumulated (cf. Fig. 1) indicates that the opened channels close spontaneously as in the case of (Ca^{2+}+ caffeine)-induced Ca^{2+} release.

c. Common inhibitors

If some reagents block the Ca^{2+} release channel and if the common channels operate in the different types of Ca^{2+} release, then such reagents inhibit all types of Ca^{2+} release (common inhibitors). We have searched such inhibitors. Ruthenium red was reported to inhibit (Ca^{2+} + caffeine)-induced Ca^{2+} release (18,19,24,27,28). We have now found that low concentrations of ruthenium red almost completely inhibit the rapid phase of depolarization-induced Ca^{2+} release (I_{50} = 0.1μM; D.H. Kim and N. Ikemoto, unpublished observation). The other types of Ca^{2+} release (slow phase of depolarization-induced Ca^{2+} release, Ca^{2+}-induced Ca^{2+} release, and (Ca^{2+} + caffeine)-induced Ca^{2+} release are also inhibited with low concentrations of ruthenium red (I_{50} = 0.4 μM). Thus, ruthenium red appears to be a strong candidate for a channel blocker, which eventually can be used as a marker to identify the Ca^{2+} release channels.

D. Conclusion

Recent advances in the methods for the kinetic studies of Ca^{2+} release have allowed us to induce and monitor the rapid Ca^{2+} release from SR in vitro. Some new insights into the molecular mechanism of Ca^{2+} release have been gained as summarized in Fig. 4. There are several different classes of receptor for the triggering signals. They are distinguishable by their kinetic properties and by their locations. However, not more than one class of Ca^{2+} release channel is required to account for the kinetics of Ca^{2+} efflux from SR. Some experiments described in this article, such as dissociation and reconstitution of the T-tubule/SR linkage, and demonstration of the involvement of Ca^{2+} antagonist binding components in the communication route, have opened new possibilities for future studies of the molecular pathway by which the impulse elicited in the T-tubule membrane is transmitted to SR in order to trigger Ca^{2+} release from SR.

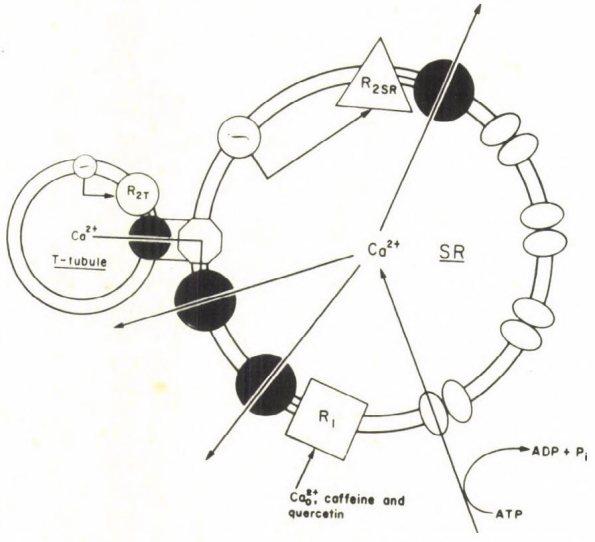


Figure 4. Schematic representation of the proposed triggering mechanism of Ca2+ release and locations of receptors and channels.

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DISCUSSION

KOVÁCS:

What physiological evidence do you have for the presence of Ca channels on T-tubular membrane. Are they voltage gated ones or not?

On the other hand, there are evidences of the location of voltage controlled Na-ions. Can you rule out a possible regulatory role of sodium ions on fast R_1 receptors?

IKEMOTO:

Since at the moment we do not have evidence that ionic replacement produces the depolarization of the T-tubule membrane potential. Such experiments will eventually permit us to examine whether the nitrendipine binding T-tubule Ca^{2+} channels are in fact voltage-gated. We are planning to carry out possible role of monovalent cation channels in the triggering of rapic Ca^{2+} release with the use of appropriate channel blockers.

DAMJANOVICH:

Comparing the kinetics of Ca^{2+} release induced by depolarization you found two rate contants. However, the amplitudes were also significantly different. Would you mind commenting it?

IKEMOTO:

This question is related to our recent finding that faster Ca^{2+} release produced with the aid of cation ionophore such as valinomycin tends to show smaller amplitudes of Ca^{2+} release. This is because wider opening of Ca^{2+} channels is followed by faster closing of Ca^{2+} channels. This interesting phenomenon may be related to the understanding of the mechanism by which traffic control of the Ca^{2+} movement operates.

KEPES:

Is there an electrostatic feedback? Is there an anion known to flow along?

IKEMOTO:

Little is known so far. However, it is very likely that compensatory changes of membrane potential such as overshoot may be involved in the control of the behaviour of Ca^{2+} channels.

MARTONOSI:

A number of investigations presume that the so-called "depolarization" induced Ca^{2+} release produced by ion substitution contains a large component that is attributable to osmotic lysis of the vesicles, and therefore is not expected to have physiological relevance.

IKEMOTO:

The rate constant of the fast component of depolarization-induced Ca^{2+} release is significantly higher than those reported in the literature. Therefore, it belongs to an entirely new category. Careful examination of the possibility that osmotic changes might affect the kinetics of Ca^{2+} release is an open question.

KÖVÉR:

Dantrolene is a well-known and good inhibitor of E-C coupling. Have you looked at its effect on potential dependent Ca release of SR?

IKEMOTO:

In view of the growing interests in the effects of dantrolene-Na on E-C coupling of the intact fiber, we have already carried out the type of studies you mentioned. Interestingly dantrolene-Na has a dual effect on the rapid phase of depolarization-induced Ca^{2+} release. At the lower concentration range it activates Ca^{2+} release, whereas at higher concentrations Ca^{2+} release is inhibited.

LIGETI:

You spoke about Ca-channels in T-tubules, which would open during depolarization. By this means the cells should gain a considerable load of Ca from the extracellular space. To prevent Ca accumulation, there should be Ca-pumps in the plasma membrane, and they should be equally active with the SR Ca-pumps. Is anything known about this?

IKEMOTO:

At the moment, the appropriate answer to your question is my colleague Dr. Hidalgo's finding that the isolated T-tubule can pump relatively large amounts of Ca^{2+} at low Ca^{2+} concentrations. This may serve as an "aftercare" mechanism.

MARTONOSI:

Would you please describe the nature of the ion substitution and consider this problem in terms of your observations.

IKEMOTO:

We mixed one volume of 0.15 M KCl with one volume of 0.15 M choline chloride in order to partially replace K^{+} with choline^{+}. These must be much milder conditions than those generally used for triggering Ca^{2+} release.

SCHATZMANN:

The role of extracellular Ca^{2+} for EC-coupling is possibly not to be sought in Ca^{2+} entry into the cell. Contractures in Ca^{2+}-free medium irreversibly destroy the ability of the muscle to contract even if 25 min are allowed for recovery in normal solution (F. Graf). This effect is not due to the loss of intracellular Ca^{2+} nor to the failure of the plasma membrane to repolarize. It might be due to the loss of Ca^{2+} from the T-tubular membrane.

IKEMOTO:

Thank you for the comments.

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KINETIC STUDIES ON Ca COMPARTMENTS OF FROG STRIATED MUSCLE

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It is an old aim in the research of muscle contraction to determine the space distribution of calcium. Assuming steady state system - in which the muscle is during our experiments - the distribution of calcium ions is homogeneous and reaches the equilibrium very fast within the phase borders. Through the phase borders - between the compartments - the transport of Ca^{2+} is "very" slow, and can be characterized by kinetic constants. It is a reasonable assumption that in steady state system the netto flux between the compartments is zero and the corresponding kinetic constants are independent of time. We assumed parallel compartments resulting in superposition of exponential functions as corresponding equations. Generally in such kind of experiments only the total amount of ^{45}Ca located in the muscle is measurable. Additionally, due to the tolerance of muscle, the duration of the measurement and the radioactivity of the medium are limited.

In our experiments a continuous flow cell was introduced, in which the muscle was fastened up to 130 % of tensionless length and its tension continuously monitored in order to check the physiological state of muscle during experiments. Any significant change of tension resulted in discarding the data of the particular muscle.

Experiments were carried out using frog m. semitendinosus in a buffer system having the composition: 115 mM NaCl, 2.5 mM $CaCl_2$, 5 mM histidine; pH. 7.0.

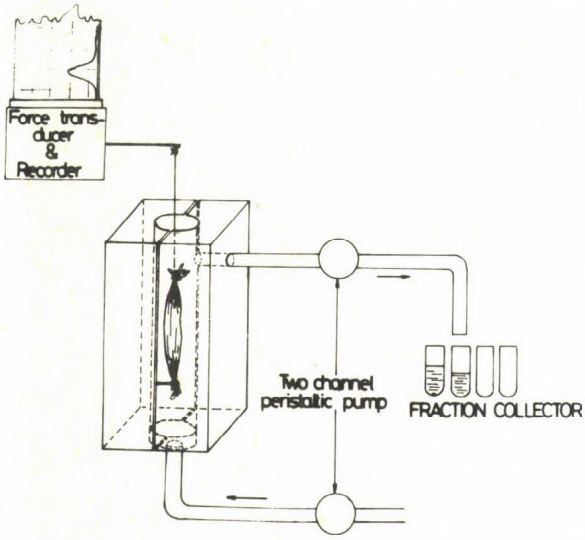

Fig. 1: Schematic diagram of continuous flow system. The two-channel peristaltic pump pushing and pulling the solution in and out resulted in a time independent volume of "outside" reservoir. During the uptake the two ends were short circuited using a special valve; and approximately 1 ml of incubation medium was flowing in the circle.
Desaturation was started by switching the valve, which means that a "cold" n-Ringer solution flowed through the cell and was collected using a programmable fraction collector.

Muscle was prepared at room temperature, the wet mass of muscle was measured and placed into the incubation chamber having an inner volume of 200 µl, approximately. The wet mass of fibers varied between 25-55 mg each. The incubation chamber was made using special plexiglass, the suspender thread was extra thin acryl and silicon tubes were used for the flow system in order to avoid any source of error due to the Ca adsorption on the wall of the experimental set up.

In the flow cell the muscle was incubated in the buffer - in the presence of $^{45}Ca^{2+}$ with approximately 2 μCi/ml specific activity for the required uptake time - short circuiting the circulation system.

Desaturation curves were obtained by opening the short circuit circulation and the hot buffer replaced by a cold one, which had exactly the same composition except that it contained non radioactive Ca^{2+} instead of $^{45}Ca^{2+}$. Fractions were collected according to the program resulting in samples having radioactivity depending on the speed of Ca^{2+} exchange between muscle and incubation medium. The influence of radioactivity of incubation medium on the muscle - because of continuous flow - can be assumed to be zero through the whole experiment.

After the measurement had been completed the muscle was digested using perchloric acid-hidrogen peroxide incubation at 95°C followed by evaporation at 130°C and the residue dissolved in n-Ringer. The radioactivity of sample fractions and of the muscle at the end of the experiment was determined using Nuclear Chicago Mark II liquid scintillation spectrometer setting, standard error 0.1 %.

From these data - corrected for blank - the radioactivity of the muscle was calculated as a function of time: this is the f(t) curve, expressed in mmoles Ca/kg muscle wet weight. The characteristic parameters of compartments are the kinetic constant which characterizes the movement of the material and, the other, which characterizes the amount of material located in it. We assumed parallel and independent compartments, which resulted in a good reproducibility and proved to be useful - so far -, however, it is known already that this assumption deviates more or less from the real situation. This means that Ca^{2+} flows from the reservoir into the different compartments and reverse but not between them. If k_{oi} represents the kinetic constant of ion transport between the i-th compartment and outside reservoir (i.e., the incubation chamber); and q_i represents the ion amount in compartment No. i, then - assuming that the cross transport constants (k_{ij}) are all zero - the change of ion amount (q_i) in time is the following:

$$\frac{dq_i}{dt} = - k_{oi} \cdot q_i$$

Applying the adequate equation for all compartments, the solution of the resulted differential equations can be written in the following form

$$q_i(t) = \sum_{j=1}^{N} B_{ij} \exp(-\lambda_j t)$$

where λ_j-s are the eigenvalues of the matrix of rate constants $[k_{ij}]$ and $\vec{B}_j = B_{1j}, \ldots, B_{Nj}$-s are the corresponding eigenvectors. If $A_i = \sum_{j=1}^{N} B_{ij}$ then the total ion amount in the system versus time can be expressed in the following form:

$$q(t) = \sum_{i=1}^{N} A_i \, e^{-\lambda_i \cdot t}$$

Due to physiological and technical reasons the $q(t_j)-q(t_{j+1})$ differences were measured and then the $q(t_o)$, $q(t_1)$, ... , $q(t_n)$ values were calculated. Deconvoluting the $q(t)$ function we get the number of resoluble compartments and their parameters. According to the hypothesis of parallel compartments, the value of λ_i indicates the rate of flow of ion between the i-th compartment and the incubation medium; A_i represents the ion amount that was taken up by the i-th compartment.

The Fourier transform method was used for the deconvolution of superposition of exponentials and for the determination of kinetic parameters, because it can generally be used in the case of theoretically infinite number of components and it is not necessary to give an approximate value for the parameters, and it is less sensitive for experimental and/or stochastical errors than the widely used methods such as the linear or non-linear least square or Peeling method.

The number of components should not be set in advance but it followes from the calculation itself without any assumption.

The price of these advantages is the difficulty of programming and the ten to twenty times longer running time of calculation compared to other methods.

The theoretical considerations and program flow chart were published earlier (Jóna and Kéri-Fülöp, 1981). The deconvolution program was running on PDP 11/34 computer (64 k) using Fortran compiler and it had a running time of about 5-8 hours depending on the individual experiments (for 60-70 data points). An individual desaturation curve is plotted in Fig. 2.

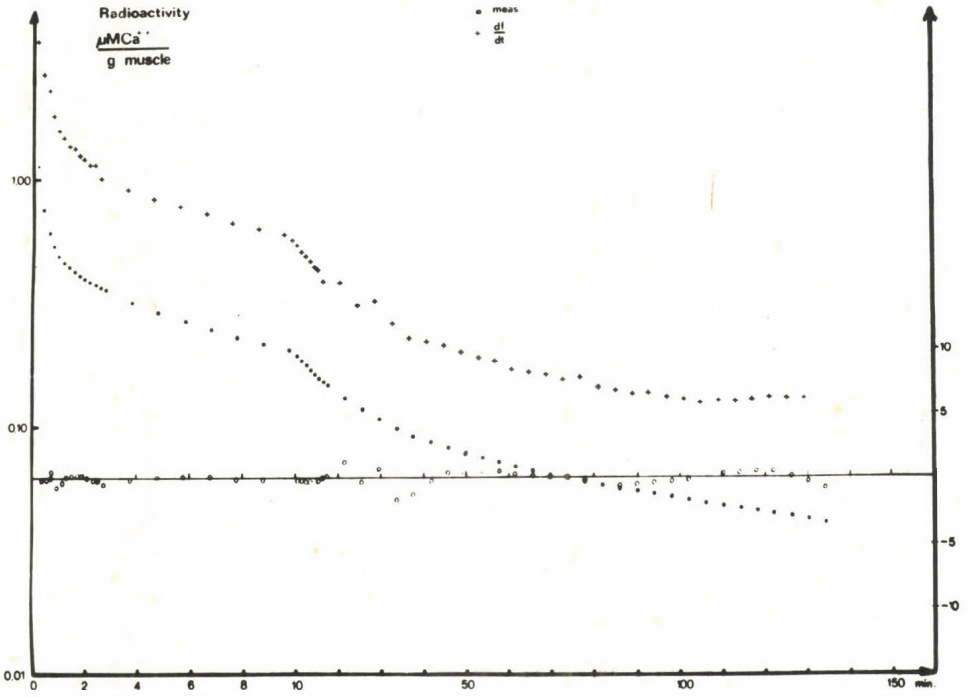

Fig. 2: Semi logarithmic plot of a ^{45}Ca desaturation curve of frog m. semitendinosus (120 mins uptake). Closed circles represent the amounts of ^{45}Ca located in the muscle and the points of the fitted curve. Crosses represent the derivative of the fitted curve in arbitrary units (left scale). Open circles represent the difference between the measured curve and the fitted curve - expressed in percentage of the measured value (right scale). For experimental reasons the 0-10 min interval is plotted on expanded time scale.

Table 1

Averaged kinetic parameters of Ca^{2+} transport in m. Sartorius bundles, using different uptake times

Comp. No.	$^{45}Ca^{2+}$ uptake time 5′	20′	60′	120′
	Time constants (min)			
1	133.5	124.8	135.7	160.1
2	60.7	46.0	60.2	22.9
3	11.2	5.4	12.0	13.1
4	2.38	1.86	1.80	2.53
5	0.316	0.317	0.318	0.414
6	0.0692	0.052	0.0671	0.070
	"Volume" of compartments (% of total exchangeable Ca^{2+})			
1	16.99	12.9	10.3	17.4
2	12.7	9.5	11.6	15.9
3	29.4	36.4	8.7	14.9
4	21.6	22.3	24.4	22.4
5	9.97	18.5	27.8	16.2
6	9.32	0.4	17.1	13.3

Each value belonging to uptake time is the average of 4-6 independent experiments. Exchange conditions are identical. Incubation medium was n-Ringer solution and volume data were extrapolated for full saturation.

The derivative function is also plotted because it characterizes the rate of Ca^{2+} flow. Any damage of the muscle can be recognized by breaks on the derivative function. In normal cases it should be smooth. The deviation plot also implemented and showed a good fit exceeding 1 % only in few cases. As a result of complicated mathematics of data evaluation the first question to arise is whether these kinetic parameters are reproducible or not. It is a good way to show the power of the method taking several different muscle fibres and carrying out experiments. Each experiment means different fibre bundles

obtained from different animals. In such a way, by repeating the experiments several times and using different $^{45}Ca^{2+}$ uptake (incubation) time, data can be obtained on the reproducibility of the whole experimental and evaluation method. These data are presented in Table 1. As it shows there are some deviations in the time constants of the compartments. Taking the "biological" deviation and the excellent fit between the measured and calculated curves into account it is obvious that the rate constants are independent of the $^{45}Ca^{2+}$ uptake time in our system. Not so excellent but a reasonably good agreement can be seen in the case of $^{45}Ca^{2+}$ content of the compartments (called: "volume"). The larger differences are probably due to physiological reasons - these parameters are not so well controlled and strongly dependent on the amount of movement of the animal from which the muscle was taken.

These data show in general, that our method gives the possibility to follow the changes of compartments in different muscles under the influence of drugs or chemicals - in steady state system.

Our first choise was the effect of lanthanium on Ca^{2+} comparments because of the well known properties of La^{3+} as well as the role of La^{3+} as "supercalcium". La^{3+} does not penetrate through the membrane but binds on the surface at the Ca^{2+} binding sites with high affinity, resulted La^{3+}-protein complex on the surface instead of Ca^{2+}-protein complex. This is a surface effect but due to the complicated geometry of the surface and the different couplings it can result in influencing other compartments, too. The kinetic parameters of muscles in the presence of 1 mM lanthanium can be seen in Table 2. As the data show, there are changes only in the two inner compartments from the point of view of time constants (rate of Ca exchange). The influence of lanthanium on the Ca-protein interaction is largely due to the high ratio of the specific binding of Ca in these compartments. The rate of exchange diffusion does not change in the other four compartments. From the point of view of the "volume" the picture becomes more complicated. Except for compartment No. 6, the "volumes" change at least two-fold,

Table 2

Averaged kinetic parameters using different times of uptake on frog m. Sartorius bundles effect of 1 mM La^{3+}

i	Time constants (min)		Amount of exchangeable Ca^{2+} (extrapolated to saturation)	
	Control	La^{3+}	Control	La^{3+}
1	138.5 ± 8.2	710 ± 41	14.4 ± 1.7 %	61.3 ± 4.4 %
2	47.5 ± 8.2	25.3 ± 2.5	12.4 ± 1.3 %	4.5 ± 0.1 %
3	10.4 ± 1.7	9.1 ± 3.1	22.3 ± 5.6 %	5.4 ± 0.2 %
4	2.14 ± 0.2	2.59 ± 0.6	22.7 ± 0.6 %	9.6 ± 3.6 %
5	0.314 ± 0.024	0.314 ± 0.005	18.1 ± 3.2 %	8.7 ± 1.0 %
6	0.065 ± 0.004	0.05 ± 0.01	10.0 ± 3.1 %	0.6 ± 1.0 %

The control means n-Ringer incubation. The effect of La^{3+} was measured in the same way except that the uptake medium as well as washing medium contained 1 mM La^{3+}. (Mean ±S.D.) Number of muscles was 8 (control) and 6 (effect of La^{3+}). The time of uptake was 120' and the apparent volume of compartments were extrapolated for infinite time of uptake and expressed as the percentage of total exchangeable Ca^{2+}.

in such a way that the four middle compartments decrease two to four-fold and in balance the most inner one increases about four-fold. These phenomena are due to the increased binding of Ca^{2+} in compartment No. 1 and in that way - under the influence of La^{3+} - a new equilibrium of Ca^{2+} manifested resulting in different Ca^{2+} concentrations in the compartments, because of competition of La^{3+} and Ca^{2+} on the surface membrane, and because of "broken" Ca^{2+} transport on the surface (Fig. 3).

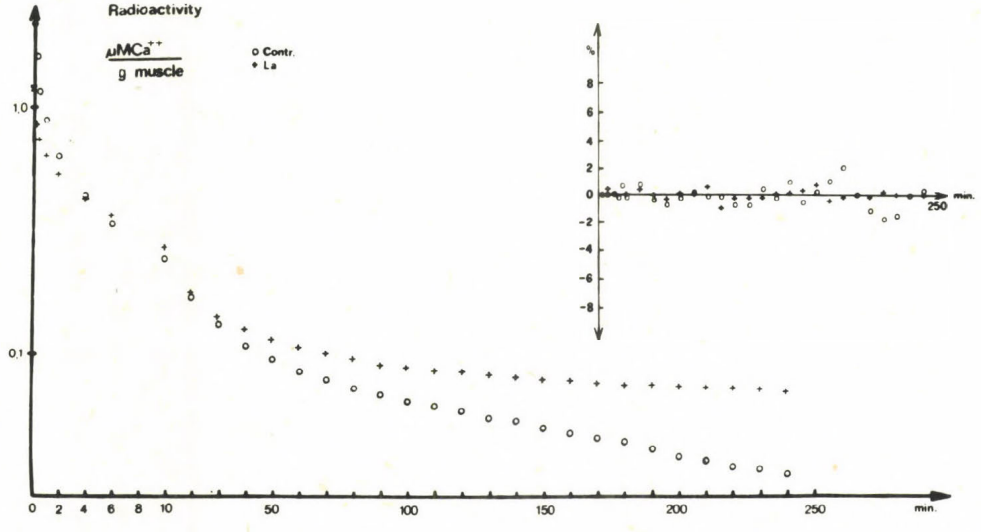

Fig. 3: Effect of La^{3+} on the ^{45}Ca desaturation curve (60' uptake). Open circles represent the amount of Ca^{2+} in the muscle and crosses represent the radioactivity in the muscle in the presence of 1 mM La^{3+}. The insert shows the deviation plot expressed in percentage of the given time total.

The caffeine and dantrolene are well known agents, which influcence the Ca^{2+} movement and the electro-mechanical coupling system. At a caffeine concentration of 1 mM - which is well below the contraction threshold (≅2.5 mM) - the effect of caffeine is reversible and using repeatedly drug free incuba-

tion medium it can be removed and the state of muscle completely restored from the point of view of the membrane potential as well as muscle contraction. This means that in that concentration the caffeine acts on the membranes in a non-specific way. (We found two compartments which were influenced.)

The dantrolene is known as a decoupling agent which influences the electro-mechanical coupling.

The effect of caffeine and dantrolene on the desaturation curve can be seen in Fig. 4. The corresponding kinetic parameters are summarized in Table 3.

Comparing the time constants in the presence and absence of caffeine there are significant changes in two compartments. Compartments No. 1 and No. 4 have about 2-3 times larger time constants showing the slower exchange diffusion and/or decreasing number of the available channels for Ca^{2+} transport. Dantrolene has similar effect on compartment No. 4 and an opposite one in the case of No. 1 because it causes decoupling and, as a result, a faster Ca^{2+} transport into the influenced compartments. The data show how the resulted new equilibrium will set the kinetic parameters. The tremendous increase of the volume of compartment No. 1 and the great increase of compartment No. 4 have the same meaning, and show the increased Ca^{2+} binding capacity as well. However, part of the second effect can be due to the overall swelling of the muscle during caffeine and dantrolene treatment. Another interesting effect is that in the case of dantrolene the outer compartment is the sum of the two outer compartments of the control. The drug made a new compartment of the two old ones, without effecting the corresponding "volume".

Taking everything into account, we had always six components, however, the faster component is not considered to have real biological meaning but to belong to the muscle surface and the limited diffusion of Ca^{2+} in the tubular system; but in order to obtain correct deconvolution it must be taken into account, and only after the calculation has been completed, can it be disclosed.

Table 3

Effect of 1 mM caffeine and 0.5 mM dantrolene on kinetic parameters (frog m. semitendinosus)

Comp.	CONTROL	DANTROLENE	CAFFEINE
	TIME CONSTANT (min)		
1	110.3	61.97	208.6
2	17.9	13.34	22.9
3	2.95	3.14	4.8
4	0.56	1.32	1.54
5	0.2	0.169	0.194
6	0.089		0.061
	APPARENT VOLUME OF COMPARTMENTS (mmoles Ca^{2+}/kg muscle)		
1	0.14	0.598	0.295
2	0.123	0.212	0.055
3	0.296	0.138	0.159
4	0.20	0.449	0.30
5	0.581	1.805	0.404
6	1.33		1.47

Each column represents the average of 4-6 independent experiments. In the case of caffeine and dantrolene the uptake solution as well as the washing solution contained the drug in the appropriate concentration. Data are obtained after 60 minutes' uptake of ^{45}Ca in n-Ringer solution.

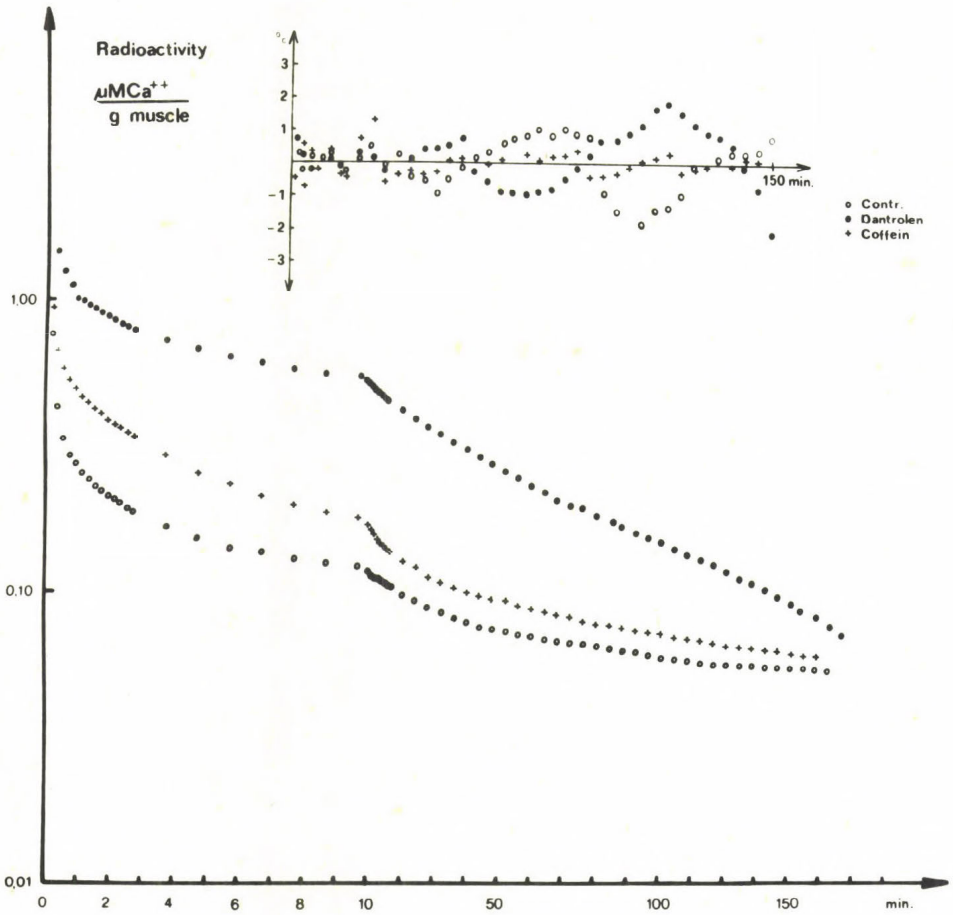

Fig. 4: Effect of caffeine and dantrolene on ^{45}Ca desaturation curve of frog m. semitendinosus. The time of ^{45}Ca uptake in each case is 120 min. Fractions were collected according to the standard procedure. Open circles represent the control muscle. Closed circles represent the radioactivity of muscle in the presence of dantrolene, and crosses have a similar meaning for caffeine. During the uptake as well as during the release the incubation medium contained 0.5 mM dantrolene (closed circles) or 1 mM caffeine (crosses).

The above data support that our newly introduced method is useful for kinetic analysis and the changes of rate constants as well as the "volume" of compartments can be followed under different conditions and/or under the influence of chemical agents. Measuring the parameters using different drugs having known points of action, these kinetic compartments can be correlated with some of the morphological and/or biochemical compartments and this way it would be possible to calculate the sapce distribution of Ca^{2+} on morphological or biochemical basis.

ACKNOWLEDGEMENTS

Thanks are due to Professor S. Damjanovich for the tremendous computer time, to Professor A. Martonosi for the valuable discussions and to Ági Kondor for her excellent technical assistance.

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DISCUSSION

RESCH:

Could you give us some of your ideas about the biological correlations of the compartments which you defined kinetically?

One of your assumptions was, that all compartments are in exchange with the outer space. Is that feasible if we assume that some compartments could be in intracellular organelles, e.g., mitochondria?

JÓNA:

All of these experiments were carried out on frog skeletal muscle which is a white muscle and all the mitochondria are located near the outer surface, additionally this white muscle does not have such a large amount of mitochondria which can contribute in the calcium distribution of the muscle to a measurable extent compared with the sarcoplasmic reticulum or other specialized calcium binding compartments. However, I cannot exclude that one of the compartments demonstrated by us represents the mitochondria or other intracellular organelles. Besides I should like to emphasize that we measured kinetical compartments and not morphological ones.

FONYÓ:

I am not that sure that you can reject mitochondria off hand as although the amount of mitochondria is small in your muscle their capacity for Ca^{2+} might be large.

JÓNA:

I do not reject it. As I said - one of the compartments might correspond to the mitochondria.

SCHINDLER:

Have you employed other tissues in your study to examine the specificity of the compartmental analysis for muscle? For example liver lobe, kidney?

JÓNA:

No, I have not. The main aim of our experiments was to develop such technical and mathematical methods which make it possible to carry out kinetical compartment analysis on whole, "untouched" muscles without any heavy treatment. In case of any kind of slices - because of injuries of the surface - additional compartments may form, increasing the sources of error.

IKEMOTO:

Some complications in the analysis of the data may have arisen from the fact that Ca^{2+} release is restricted by the plasma membrane permeability barrier. Is not there any possibility of extending similar type of studies with the "skinned" muscle fibers?

JÓNA:

You are right in that respect that any membrane can restrict ^{45}Ca release being a permeability barrier. But this makes possible - among other factors - the compartmentalization study itself as the desaturations are influenced by them. Similar experiments can be made with skinned fibres too, but we have not tried it yet.

SCHONER:

I will come back to Dr. Resch's question and will ask it in a different way. I understood that you did not study the compartments in isolated single cells. Because you showed in your first figure a recorder on top of your experimental set up, I would like to ask you whether you did some tentative studies to define, by biological experiments, the meaning of your compartments.

Did you understand you were correct that mitochondria do not form a compartment in your system?

JÓNA:

We have an idea that certain kinetic compartments may correspond to one or some morphological ones. By the analysis of the effect of different pharmacological agents (e.g. dantrolene, caffeine) both the functional and the morphological identifications of compartments can be performed. On the basis of our experimental data we suppose that the most inner compartment corresponds to that of SR. The second one means obviously the calcium bound to the cytoplasmic surface of SR. Compartment No. 3 is the cytoplasmic surface of the T-tubuli, and compartment No. 4 is the luminal surface of the tubuli. Compartment No. 5 corresponds to the space of T-tubuli and No. 6 is the extracellular space among fibres as well as other undistinguishable components. Compartment No. 4 and No. 5 obviously include also the internal and external surface membrane bound calcium. Mitochondria can form a compartment, but we cannot measure it so far.

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POTASSIUM PATHWAYS IN ESCHERICHIA COLI

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INTRODUCTION

Prokaryotes and Escherichia coli in particular do not possess (Na, K) ATPase in their plasma membrane, like higher eukaryotes do. Nevertheless K^+ is the predominant cytoplasmic cation and it is essential for growth. The reason of the absolute requirement for potassium in prokaryotes, as in eukaryotes, is not entirely understood. One role of potassium is, however, probably specific to bacteria, namely the necessity to maintain an osmotic pressure in the cytoplasm exceeding the osmotic pressure of the medium, so that a hydrostatic pressure, the turgor pressure, keeps the plasma membrane in close contact with the rigid murein layer.

The importance of potassium is underlined by the number of mechanisms which concur to its regulation. This multiplicity of mechanisms, the complexity of the regulation make the study of potassium pathways in prokaryotes relatively unattractive, so much so that the molecular basis of the potassium fluxes in higher organisms has largely been elucidated earlier. Few groups devoted sustained attention to the problems of potassium uptake in prokaryotes. Most of what is presently known of the K^+ pathways is due to the work of the group of W. Epstein. They established the map of genes involved in potassium metabolism, characterized the functions of important gene products, notably they identified and isolated, the membrane-bound K^+ ATPase. (For a review see Silver, 1978; Helmer et al., 1982.)

In the following an attempt is made to describe potassium fluxes in bacteria with the aim of illustrating some problems of general importance open to further investigation rather than to give an exhaustive account of accomplishments.

Obtention of potassium-free cells. A mechanical disorganization of the hydrophobic continuum

Since potassium is essential for growth, bacteria harvested from a culture possess a normal pool of potassium 0.1-0.2 M, in the cytoplasm. Resuspended in a sodium-based potassium-free medium, after a small initial loss of 15-25 per cent they lose intracellular potassium at a rate of about ten per cent per hour. The addition of valinomycin was utilized extensively to deplete potassium fast and thereby create an electrical potential in membrane vesicles, but in whole bacteria due to the barrier opposed by the outer membrane to the access of the inner membrane, the effect of valinomycin is somewhat delayed and higher concentrations of the ionophore are required. Cells depleted with this method remain permeable to K^+ and they are not an optimal starting material to explore the K^+ pumps.

One physiological means to deplete K^+ would involve the utilization of the K^+/H^+ exchanger described by Rosen (Tsuchiya and Rosen, 1976; Brey et al., 1980; Plack and Rosen, 1980; Sorensen and Rosen, 1980). This "antiporter" is believed to be essential for the regulation of intracellular pH and therefore to be essentially active at alkaline pH in the H^+ in K^+ out direction. In reality potassium efflux was described in tris-buffered alkaline media at pH values where a $tris^+/K^+$ exchange could become operative, instead of a H^+/K^+ exchange. In order to check this hypothesis, K^+ depletion was measured as a function of pH and of tris concentration, keeping the osmotic pressure of the medium constant. In the whole range the displacement of potassium correlated well with the concentration of tris base in the medium. Replacement of tris buffer by triethanolamine buffer or by imidazole buffer changed the pH of half maximal K^+ displacement in accordance with the respective pK values of the buffering species, indicating again a correla-

tion with the concentration of the base form rather than the pH (Meury, unpublished).

Another way to deplete cell potassium is to inhibit the energy generation process necessary for its accumulation. 2,4--dinitrophenol was used for this purpose at a concentration of 10 mM (Rhoads et al., 1976). This is 10-50-fold of the concentration necessary to deplete cytoplasmic solutes accumulated by protonmotive transport system. At these concentration the proton conducting uncoupler might play the role of a K^+ ionophore (Kessler et al., 1976).

The method applied in the authors' laboratory is the sudden decrease in the osmotic pressure of the cell environment, i.e. an osmotic "down-shock" (Tsapis and Kepes, 1976; Kepes et al., 1977). The method can be used after centrifugation by resuspending the pellet in a solution of low osmolarity, or after filtration, employing very dilute aqueous solutions, or even distilled water for washing. When the osmolarity both of the suspending fluid and the washing solution was varied, half depleting shocks were obtained with washing fluids whose osmolarities were directly related to that of the suspension fluid to which bacteria had previously been adapted. The drop of osmotic pressure necessary to attain this result was roughly 0.4 osM. Bacteria adapt to an osmolarity different from that of the culture medium in a few minutes and as shown below the adaptation is essentially due to the adjustment of the intracellular K^+ pool.

The leak of K^+ caused by the osmotic shock is however not part of a physiological adaptation process. It is due to a momentary breakdown of the osmotic barrier of the plasma membrane, which regains its normal impermeability a few seconds (and maybe in fractions of one second) later. The leak is not specific, sugars, aminoacids and nucleotides also leak out during the shock and probably non-penetrant solutes can be introduced into the cytoplasm if present in the washing fluid (Haguenauer-Tsapis and Kepes, 1980). The leak is restricted to small molecules, no TCA precipitable material is released. These observations rule out both a physiological regulatory

response of the cells to the osmotic shock which is based on solute recognition and a gross damage of the membrane that requires more time to be repaired and can also release macromolecules. We therefore proposed a more subtle physical modification of the membrane. The principal characteristics of this change relied on water uptake, causing a sudden increase in the turgor pressure. Such a turgor pressure would press the membrane against the rigid murein layer and this anisotropic transmembrane pressure might dislocate the bilayer structure, until, after a short time of relaxation, the pressure becomes again isotropic and the organization of the bilayer reestablishes the hydrophobic continuum. A support for this relaxation of the bilayer comes from the observation that osmotic shocks are more effective at low temperature than at 37°C although the correlation with the transition temperature of the membrane lipids has not yet been established.

The hypothesis of a modification of the bilayer structure that it becomes permeable to hydrophilic solutes under a transient anisotropic pressure is largely speculative and it is based on the known changes of the turgor pressure and the known effect on the release of solutes. Actually such an anisotropic pressure is difficult to produce experimentally otherwise than owing to a rigid envelope. There is no more possibility to come to mind, except for the electrostriction effect on planar bilayer membranes, which occurs for transmembrane electrical potentials of the order of 300 mV and causes an instantaneous breakdown of the electric resistance (Requena et al., 1975). Speculation could be extended to the observation of the formation of aqueous channels in artificial bilayers at very low pH. The mechanical effect here envisioned, namely a repulsion of the electric charges of the same sign on the polar heads of the two sides of the membrane is geometrically opposite to the supposed effect of increase in turgor or in Coulombian attraction. Maybe, the three phenomena can be grouped into a class of mechanical effects at an intermolecular or quasimolecular scale, having important physico-chemical consequences.

Potassium uptake and its mechano-chemical switch

The mapping of genes governing the K^+ requirement of Escherichia coli established by Epstein and Davies (1971) includes genes coding for two primary potassium pumps: the Kdp pump of micromolar affinity for K^+ is repressed in the usual high potassium media and is synthesized only under K^+ starvation. It consists of a K^+ dependent ATPase (Epstein et al., 1978; Wieczorek and Altendorf, 1979), three subunits of which have been identified on acrylamide gel electrophoresis (Laimins et al., 1978). The dp D gene is the regulatory gene of the operon and the derepression is dependent on the low turgor pressure as shown by inserting lac Z gene under its control (Laimins et al., 1981). The second potassium pump, coded by the trkA gene is synthesized constitutively and it has a millimolar affinity to K^+. Its direct energy donor is not known, both a transmembrane electrochemical H^+ ion gradient and ATP were found to be necessary for its function (Rhoads and Epstein, 1977).

The potassium pool built up by the primary pump in the cytoplasm is linearly related to the osmotic pressure of the medium, while it varies only by a factor of about 2 over a 10^5 fold change in external potassium (Kepes et al., 1977). As a consequence, the chemical gradient of K^+ ions in steady state conditions can also vary from $2 \cdot 10^5$ to about four corresponding to equilibrium potentials ranging from - 310 to -36 mV. Since the steady state membrane potential in Escherichia coli is usually in the range of -80-120 mV one can conclude that K^+ does not distribute between medium and cytoplasm according to Nernst equilibrium. In other words, K^+ uptake is not due to an electrophoretic process. Similarly, the electrochemical gradient of H^+ ions in Escherichia coli is maintained in relatively narrow limits falling between -180 and -240 mV, under physiological conditions. Therefore a mechanism of K^+/H^+ antiport would create a K^+ gradient opposite to the observed one, whereas a H^+/K^+ symport appears absurd, since this should represent a primary proton pump. The K^+ pool size fails to give an indication regarding the energy source of the TrKA pump,

and if the highest observed K^+ gradients are compatible with the K^+ ATPase mechanism of the Kdp system with K^+/ATP stoichiometry of one, the identification of the energy sources does not contribute to the explanation of the regulation of the K^+ pool. The only significant regulation which has so far appeared is realised through the osmotic pressure.

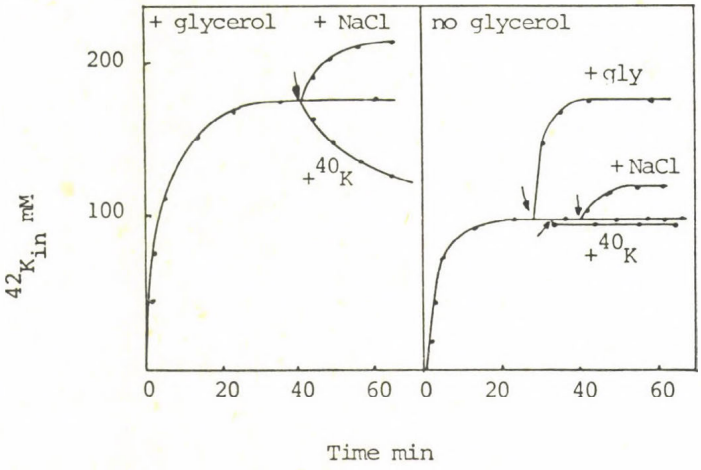

Fig. 1: Uptake, osmotic adjustment and chase of ^{42}K in the presence (left) or absence (right) of a carbon source.

Fig. 1 shows the behaviour of K^+ depleted cells when presented with 1 mM K with or without a carbon source. The rate of uptake is similar in the two cases and the attained K^+ pool differs by a factor less than two, being higher in the presence of the carbon source, glycerol. In both situations an increase of the osmotic pressure of the medium by addition of NaCl is immediately followed by a readjustment of the K^+ pool to a higher value. The duration and the amplitude of this response is also nearly insensitive to the presence of the carbon source. The same type of response is obtained by the addition of sucrose, causing the same increment in osmolarity, therefore Na^+ ion or Cl^- ion play no specific role. Instead

the effect of NaCl emphasizes the K^+ specificity of the response.

If the presence of the carbon source has only a moderate quantitative effect on the phenomena described so far, it is not so when the chase of ^{42}K by excess of ^{40}K is considered. In the glycerol-fed suspension cellular K^+ appears to be exchangeable while in the carbon starved suspension K^+ is not exchangeable.

Since in the case of many other transported solutes the steady state pool is the result of a balance between uptake and efflux (whether efflux is due to a leak or to the reversal of inward transport), it is often assumed that the steady state K^+ pool is also due to such a balance. The carbon-starved suspension of Escherichia coli gives the most convincing refutation of this belief, since at the steady state no influx and no efflux is observed. Since, as it is shown by the response to NaCl the arrest of influx is not due to the insufficience of the available energy, it is due to a regulatory arrest. Thus the signal of start of the K^+ pump is not the actual value either of the environmental or the intracellular osmotic pressure but a disturbance of the constant difference between the two. The addition of the osmoactive solute causes an immediate outflux of water across the membrane, the cytoplasm tends to shrink, the turgor pressure decreases. This switches the K^+ pump on. Together with the K^+ uptake water is entering the cell, the cytoplasm tends to swell, the turgor pressure increases. Once it reached its new value, equal to that prevailing before the disturbance, the K^+ pump is switched off. This on-off operation was made clear by the experiment on the carbon starved suspension, whereas in the carbon fed cells the K^+/K^+ exchange masked the <u>off</u> situation. The decrease in turgor pressure beyond a certain threshold can reach a stage of plasmolysis, when the shrinkage of the cell is of such an extent that the plasma membrane loses contact with murein and outer membrane. In this respect it is worth noting that most empirical procedures, which aim to plasmolyze Escherichia coli are carried out in potassium-free media (Repaske, 1958; Neu and

Heppel, 1964), thus depriving the cell from its defence response of pumping potassium in. It is very likely that the response of the K^+ pump takes place before plasmolysis because the K^+ pump responds to subtle changes in local molecular interaction. The process must be a qualified mechano-molecular action, only a small step away from a mechano-chemical transduction. It requires a mechanoreceptor at the molecular level, which can be distinct or identical with the molecule carrying out the K^+ pumping function. A direct kinetic eivdence of a similar regulation of the Kdp ATPase is not available, but the steady state regulation makes it likely that it also obeys the same kind of signal. Even the so called TrKF system seems to be capable to fulfill a limited osmoregulatory function. Since the derepression of the Kdp system is also under osmotic control (Laimins et al., 1981), a whole family mechano-receptors is conceivable.

Potassium-potassium exchange, a metabolism dependent, energy independent process

This exchange can be observed in the context of chase of ^{42}K with excess non radioactive K^+ as shown in Fig. 1 or starting with cells containing a normal amount of non radioactive potassium by the addition of trace amounts of ^{42}K without perturbation of any of the prevailing parameters of possible physiological significance. The exchange under these conditions is two-five times slower than the rate of the initial uptake, suggesting that the two phenomena might not follow the same route. The finding that the rate of exchange follows a Michaelian behaviour as a function of extracellular K^+ concentration, with an apparent K_m close to that of the uptake, via the TrKA system, suggested that TrKA was responsible for the inward flux during exchange and a leak was necessary only to release the regulatory lock of TrKA.

But Fig. 2 shows that the exchange occurs only when a carbon source is present and actively metabolized. Indeed exchange was blocked by anoxia under nitrogen bubbling in cells grown aerobically on glycerol or by cyanide when O_2 bubbling

has permitted the exchange. Under aeration glucose as well as succinate being a purely oxidative substrate, were able to support the exchange phenomenon, but glucuronate uptake in a non metabolizing mutant, AJ 19, had no effect, although its uptake system was fully active under carbon starvation.

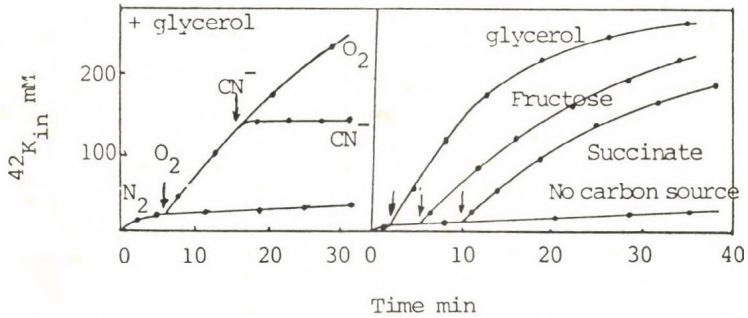

Fig. 2: K^+ exchange in potassium replete cells under a variety of metabolic conditions.

It was also shown (Fig. 1) that a carbon source helps the cell to build up a higher K^+ pool (maybe by generating anionic metabolites?) and a higher pool should lead to a leak by exceeding a hypothetic threshold of efflux.

The experiment represented in Fig. 3 makes this supposition unlikely. Potassium uptake was monitored by ^{42}K in a carbon-starved suspension and K^+/K^+ exchange by repeated 6 min chase experiments with a 50-fold excess of nonradioactive KCl. The chase was very ineffective. After the steady state had been reached, a small amount of glucose was added that could permit only five-ten minutes' active metabolism. K^+ pool started to increase immediately and at the same time chase became efficient even before maximal K^+ pool had been completed, but, more significantly, chase became inefficient again when glucose was exhausted, well before the K^+ pool dropped back to the carbon starved level. The precision on this experiment is modest because the chase periods are long enough to overlap K^+ uptake, plateau and leak periods, but the conclusion is difficult to escape, that neither the active uptake nor the high K^+ pool, but only the active glucose metabolism correlate closely with the exchange process. Indeed if the threshold of

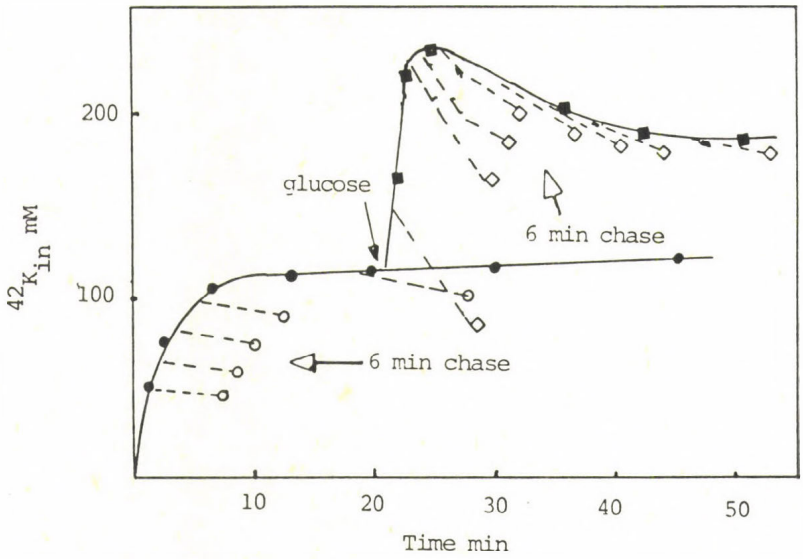

Fig. 3: Transient activation of K^+ exchange (measured by chase) during rapid consumption of added glucose

leak was exceeded by the increased K^+ pool this would tend to lock rather than unlock the K^+ pump and thereby would stop the exchange. The only ad hoc hypothesis to save the role of TrKA pump in the exchange process would be the assumption that the metabolism of a carbon source sets the pump-locking threshold substantially higher than it does during starvation and higher than the threshold of the leak, i.e., it reverses the order of the two thresholds prevailing under carbon starvation. Fig. 4 rules out even this hypothesis. The effects of two energy inhibitors were tested on uptake, exchange and steady state pool of K^+ in a medium containing glycerol.

Both, cyanide and the uncoupler CCCP lowered the steady state K^+ pool by 30-50 per cent but did not dramatically deplete the K^+ pool. Cyanide decreased the rate of uptake proportionally to the decrease of the pool, while CCCP completely inhibited the uptake. Conversely CCCP decreased the rate of exchange proportionally to the decrease of K^+ pool, while cyanide completely inhibited exchange. Let us remind that CCCP while abolishing proton motive energy permits or even stimulates (in state four) O_2 uptake. The important conclusion of this experi-

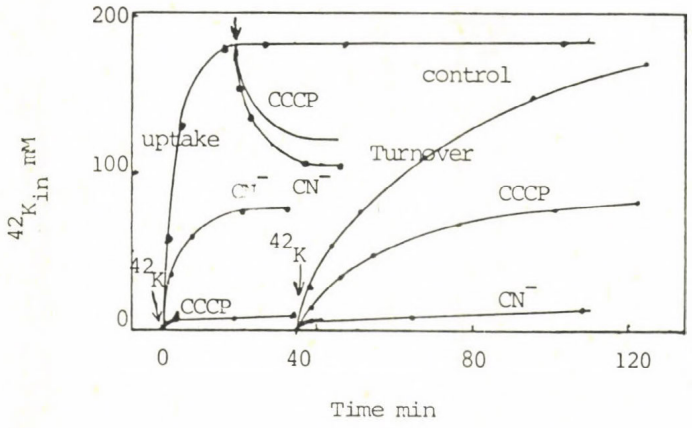

Fig. 4: Effect of two metabolic inhibitors on uptake, steady state pool and exchange of potassium in aerobic suspension with carbon source present

ment is that TrKA pump is not responsible for the inward flux during the exchange, because exchange survives the complete inhibition of uptake via TrKA. Since CCCP counteracts the effect of the carbon source in raising the steady state level of K^+ above a hypothetic threshold of leak, the role of an overflow in switching on the uptake pump also loses plausibility. Its strict dependence on oxidative metabolism is not an energy requirement. A K^+/K^+ exchange is not accompanied by thermodynamic work, this makes it plausible that it can operate in the presence of a proton conducting uncoupler.

The metabolism dependence of the exchange rather suggests the necessity of a cofactor which is an ubiquitous but short lived metabolic intermediate.

The distinctness of the metabolism dependent exchange (MDE) from the primary potassium pump is strongly supported by the experiments reported here but its molecular basis remains to be identified.

The effect of thiol reagents: A reversible opening of potassium specific channels

The addition of NEM or pCMB or $HgCl_2$ provoke a massive and complete efflux of cell potassium (Meury et al., 1980). This was reminiscent of the efflux of lactose analogs accumulated by lactose permease, where the key effect was the inactivation of the carrier. The slower effect of the slowly penetrating pCMB on the K^+ pool compared to its immediate effect on lactose permease could be understood as a different orientation of the essential -SH. But, as shown in Fig. 4, inhibitors of the K^+ pump such as CCCP do not cause a similar massive and complete leak of K^+. Even more astonishing was the finding, that by decreasing the concentration of NEM not only the leak of K^+ became slower but after some time K^+ was reaccumulated (Meury, unpublished). With optimal concentration of NEM, 0.5 mM, the addition of 2-mercaptoethanol also resulted in a rapid recovery of the K^+ pool and this was not inhibited by chloramphenicol. The loss of K^+ by addition of NEM and the recovery after adding 2-me could be repeated several times. Alkylation with N-ethyl-maleimide is irreversible, so one was led to admit that the essential thiol compound was resynthesized but that it was not a protein. It must have therefore been a cofactor of the protein which we believed at the early times to be the K^+ pump.

It was difficult to reconcile this hypothesis of the inactivation and the recovery of K^+ pump on the basis of the following observations. First, it was shown that NEM caused a massive leak, even when the K^+ pump was stopped by the regulatory switch, and the exchange mechanism made inactive by carbon starvation, or inhibited by cyanide and that the addition of NEM established both an inward and an outward flux of K^+. The second observation was made on cells in which the Kdp system was derepressed so that the cells accumulated K^+ from a medium containing 10^{-5} M KCl. The addition of NEM provoked a massive loss of K^+ like in the case of cells utilizing the low affinity constitutive TrKA system. These experiments strongly suggested that NEM opened a highly permeable potassium channel through which K^+ can freely flow both ways, instead of closing a K^+

pump which normally assured a purely inward flow. The reversal of the K^+ loss could then be attributed to the closing of these channels so that the activity of the K^+ pump(s) became capable of accomplishing an accumulatory work.

N-ethyl-maleimide has multiple effects on bacterial cells, including the inactivation of lactose carrier (Kepes, 1960; Fox and Kennedy, 1965), glucuronic acid permease (Jimeno and Kepes, 1973) and glucose phosphotransferase enzyme II (Haguenauer-Tsapis and Kepes, 1973). Nevertheless it does not destroy the permeability barrier of the plasma membrane, the solutes accumulated by lactose permease leak out at a rate dependent on the hydrophobicity of the aglycon, lactose leaks slowly, TMG faster (in conformity with their respective rate of turnover during the activity of the uptake system). Glucuronic acid leaks out at negligible rates upon the addition of NEM, its turnover under the conditions of active uptake goes entirely through the pathway of the shuttle of its carrier. Some other uptake systems are not sensitive at all to thiol reagents, e.g., substrates accumulated by gluconic acid permease, or by β methyl galactose permease, remain in the cell when thiol reagents are added. Since, among the negative findings quoted above only neutral and anionic sugars were listed, the possibility remains that cations other than K^+ and its substitutes Rb^+ and Tl^+ can also be accomodated by the channel opened by the reaction with NEM. Bakker and Mangerich (1982) showed that protons and triphenyl phosphonium ions exchange against K^+ during the opening, but definitely the cell membrane is not dramatically permeabilized.

The K^+ channel is specifically controlled by glutathione. It is a GSH controlled channel GCC

In search of the thiol compound which leaves the K^+ channel open when removed by NEM and which closes it again during recovery, it was necessary to eliminate firstly glutathione the overwhelmingly major thiol compound encountered in Escherichia coli. The concentration of glutathione is normally 1-3 mM in the cytoplasm. Mutants of Escherichia coli deficient

in glutathione synthesis such as AB7, AB821 and AB830 isolated by Apontoweil and Berends (1975a, b) appeared as the most suitable strains to start the search for the essential thiol. These mutants had growth characteristics undistinguishable from that of the wild type, in particular they did not require supplements in the growth medium such as glutathione or one of its precursors. Nevertheless inoculated into media containing 1 mM KCl, they failed to grow, contrasting with the normal growth of the parent strain (Meury and Kepes, 1982). When grown on media containing 20 mM KCl or more, upon resuspension in a potassium-free medium, they exhibited a massive loss of their K^+ pool similar to that of the K^+ leaky strains TrKB, TrKC or B525 and B526, and very different from the behaviour of wild type strains (Fig. 5). When supplemented with glutathione in the growth medium, they regained the capacity to grow on 1 mM KCl and the K^+ tight phenotype in a potassium-free medium.

It seemed therefore that GSH itself was responsible for the K^+ tightness of the bacterial membrane and that in its absence the K^+ leak occurred according to all likelihood through the same channels as those opened by addition of NEM in wild type bacteria. When the GSH-less mutants were grown on 20 mM KCl, the addition of NEM did not provoke the rapid and massive loss of K^+ observed on the wild type, but when grown with glutathion in the medium, the effect of NEM became similar to that observed in the wild type. The concentration of glutathione in the growth medium, required to obtain the two effects: growth on low K^+ and sensitivity to NEM, is less than 10^{-5} M and appreciable changes can be observed with as little as 10^{-7} M GSH. This observation rules out the possibility that the active species could be an impurity contained in GSH. The possibility that the active species is a metabolic product of GSH is made relatively unlikely by the fact that besides disulfide bond formation no significant metabolic reaction is known to occur with GSH and by the observation, that the repair of the K^+ leaky phenotype starts immediately upon addition of GSH. This can be shown as the increase of the K^+ pool upon addition of 5×10^{-5} M GSH to the mutants suspended in a medium containing 0.1 mM KCl or as the appearance of NEM sensitivity. Full effect takes

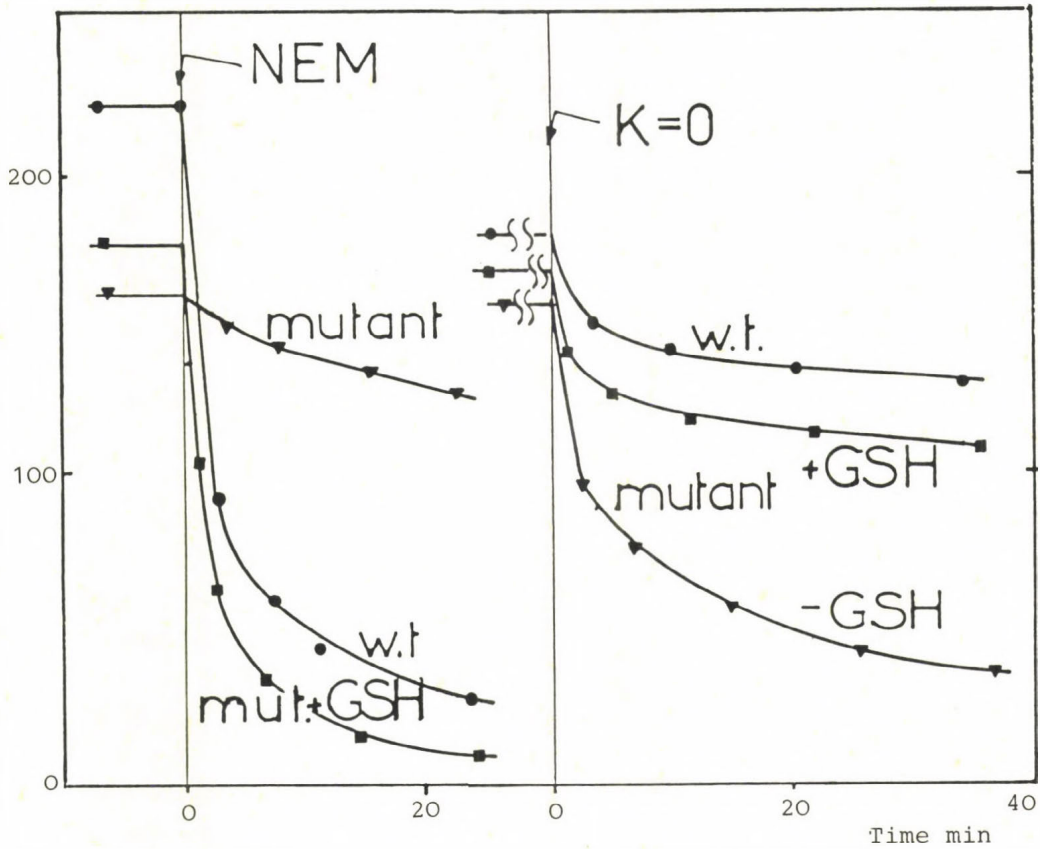

Fig. 5: Potassium loss by addition of a thiol reagent or by resuspension in a potassium-free medium of wild type (w.t.), and a GSH deficient mutant of Escherichia coli supplemented or not with GSH.

some 30 min to be completed with this concentration of GSH in the medium, and during this time an important accumulation of GSH by the bacteria occurs, achieving several hundredfold concentration ratios (Robin, unpublished).

The GSH deficient mutant, which is impaired in glutathione synthetase and which accumulates γ-glutamyl-cysteine, behaves similarly to the mutant impaired in γ-glutamyl-cysteine-synthetase. Thus, the dipeptide cannot replace the tripeptide GSH. In contrast ophtalmic acid, γ glutamyl-α-amino isobutyryl-glycine, an analog of glutathione without an -SH group is capable to replace glutathione as a supplement in the growth medium or as an additive restoring K^+ tightness in non growing suspen-

sions of the mutant, but of course it does not restore NEM sensitivity.

A number of commercially available tripeptides have been tried as substitutes for glutathione. So far only α-γ glutamyl cystin $(gly)_2$, a pentapeptide with a disulfide bond, gave positive results. After incubation with the pentapeptide the K^+ tight phenotype, but also NEM sensitivity were restored, being a probable effect of glutathion reductase on the disulfide bond. Therefore, the present tentative conclusion might be, that only GSH and its close analogs can be recognized by the K^+ leak channel, but the SH group is not essential for activity and a free α carboxyl group of the glutamic acid residue is not necessary either.

Some unsolved problems

The reason for a GSH controlled K^+ channel is far from being clear. Especially the physiological gating by (removing) GSH has no supporting evidence. A first possibility was the involvement of GCC in the metabolism dependent K^+ exchange, a process more physiological than a K^+ leak. A preliminary enquiry has been devoted to the exploration whether the glutathione supplemented and the glutathione deprived GSH mutants exhibit the metabolism dependent K^+ exchange to the same extent. The results, represented in Fig. 6 gave an affirmative answer to this question.

The level of the K^+ pool is lower in the unsupplemented cells, but its sensitivity to NEM is much lower than that of the GSH supplemented cells. The unsupplemented cells also exhibit a spontaneous non-metabolism-dependent K^+ turnover, which appears upon chase with excess non radioactive K^+. Nevertheless the K^+ exchange flux is accelerated upon addition of glycerol to the same extent as in the GSH supplemented cells, thus metabolism dependent exchange does not depend on the GCC-GSH complex.

In view of this negative conclusion it cannot be ruled out that GCC is a mere artifact, namely a glutathione binding membrane protein which changes its configuration when glutathione is removed and assumes a new configuration, which

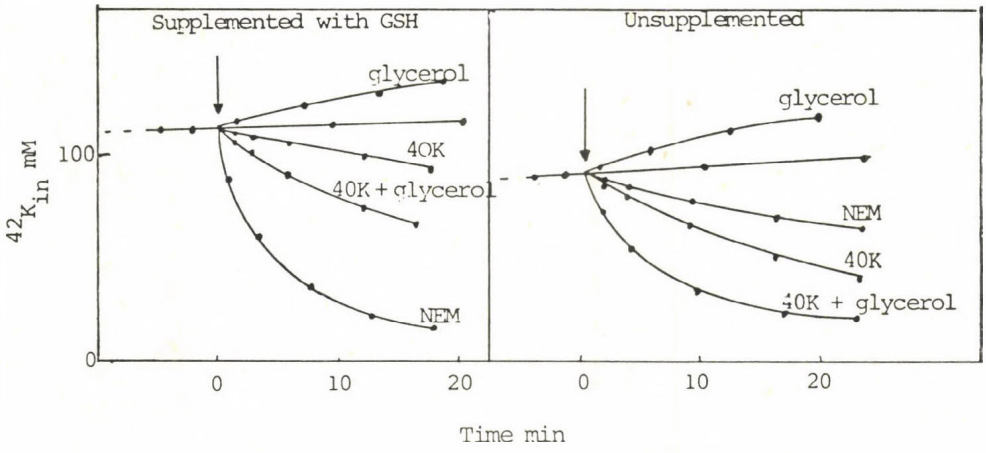

Fig. 6: Metabolism dependent K^+ exchange (MDE) in supplemented and unsupplemented suspensions of a GSH deficient mutant. MDE is the difference between the flux provoked by ^{40}K or by ^{40}K and glycerol.

fortuitously can yield passage to potassium ions. Of course the same reasoning applies to the TrKB and TrKC gene products, which could be genuinely unrelated to potassium pathways of Escherichia coli but become K^+ channels due to a genetic damage. In this case these gene products as well as the GCC could still serve as model systems insofar as they authorize new physicochemical, structural or genetic approaches, less easily available with the physiologically significant channels. In this respect the glutathione controlled channel has the advantage of the reversibly controlled opening and closing by experimentally adding glutathione or thiol reagents.

Inversely, it is possible that GCC is a physiological cation channel, which can be gated by influences other than GSH binding-dissociation, to be discovered. It is also possible, that its physiological role is to conduct something other than K^+ ions, for example Ca^{2+} ions.

These questions are not the only ones connected with the K^+ pathways of Escherichia coli. We showed above that the metabolism dependent K^+/K^+ exchange also requires a physiological role and the elucidation of its molecular mechanism.

The TrKA system itself, working with an unknown energy source, is controlled by a vaguely understood mechanochemical transduction and maybe for this reason was not so far amenable to function in isolated membrane vesicles.

The K^+/H^+ exchanger, which was explored instead only in inverted membrane vesicles, was thought to have the role to regulate intracellular pH in alcaline media, but by doing so, this electroneutral exchange should transport H^+ ions and K^+ ions, each against their respective concentration gradients and needs therefore an additional energy coupling mechanism. Moreover, if the TrKA system is clearly responsible for adjusting the K^+ pool upward in response to an increase in the osmotic pressure of the medium, the downward adjustment following a decrease in osmotic pressure still has to await for the identification of a pathway.

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DISCUSSION

WOJTCZAK:

Is there any tightly bound glutathione in E. coli membrane? In other words, does glutathione or ophtalmic acid exert their effect in the membrane itself or via some intracellular processes?

KEPES:

We have definitely found high affinity glutathione binding sites sedimenting with the cell envelopes. This binding is inhibited by the glutathione analogs which can also replace glutathione in restoring potassium retention.

CARAFOLI:

Are there any data available on the properties of the K^+ channel, with particular respect to what is known of the properties of K^+ channels in eukaryotic cells (conductivity, time of opening and closing, effect of drugs, etc.)?

KEPES:

Unfortunately E.coli is too small to stick an electrode into it. We cannot explore kinetics below the time scale of biochemists i.e., a few seconds. Concerning the effect of drugs, preliminary experiments tend to show an inhibition of K^+ efflux upon a decrease of external osmotic pressure by tetraethyl ammonium.

KÖVÉR:

Did not you use Ca^{2+} ionophore for increasing Ca^{2+} concentration intracellularly, which could increase K^+ loss according to Gáros's effect on red blood cells?

KEPES:

No, we did not start exploring the Ca^{2+} channel hypothesis and we want to explore Ca^{2+} fluxes primarily in the ab-

sence of ionophore. This raises the problem of how to get rid of the ionophore.

FONYÓ:

Just a technical comment: it is easy to use the Ca-ionophore and to get rid of it by extensive washing with albumin as shown by Sarkadi, Gárdos and Szász. In the presence of the ionophore one can get either Ca^{2+} in or out and subsequently the ionophore is removed completely by washings in albumin solutions.

KEPES:

Thanks for this good suggestion.

SOMOGYI:

In many types of mammalian cells a γ-glutamyl-cycle is demonstrated which may play a fundamental role in the aminoacid transport and uses GSH as substrate. Are there any data about the existence of this cycle in bacteria? Is amino acid transport K-dependent in bacteria?

KEPES:

Bacteria have a dozen of different aminoacid transport systems, about half of these is dependent on the proton-motive force, the other half includes the so-called shock sensitive transport systems which include, besides a transmembrane transport protein, a periplasmic binding protein. The energy source for the latter comes from phosphate bond energy, the particular energy donor molecule is still unknown. There is also one transport system, glutamate permease, which is sodium dependent. No transport implying the γ glutamyl cycle is known in bacteria.

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SUBCELLULAR LOCATION AND DISTRIBUTION OF ALKALI CATION PATHWAYS IN VARIOUS TYPES OF MUSCLES

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In the skeletal and heart muscle, as in other cells, the active transport of Na^+ and K^+ across the sarcolemma is essential for the maintenance of a wide variety of cellular properties and functions. Primarily, the Na-K-pump builds up a steady-state with a low intracellular Na^+/K^+ concentration ratio, which is controlled by the inherent kinetic properties of membrane bound Na-K-ATPase. The specific transport enzyme, the Na-K-ATPase, consists of two large subunits that span the membrane and two smaller glycoprotein subunits that are exposed on the outside of the cell membrane.

There are considerable evidences that ions can move along their electrochemical potential gradient across the membrane through voltage-gated aqueous pores called ionic channels, which also span the entire membrane (Stefani and Chiarandini, 1982), and by means of carrier molecules as well, which may shuttle back and forth across the lipid bilayer.

Skeletal muscle and myocardial cells are enclosed by sarcolemma which defines the boundary between the intra- and extra-cellular space. Portions of the extra-cellular space, however, are carried into the interior of the cell with the transverse tubular or T-system, which opens freely to the extracellular space. Both the sarcolemma and T-tubules membranes act as a barrier and in addition these membranes contain ion pumps, ionic channels and exchange sites.

Therefore it is natural to ask whether the pumping sites and passive ionic pathways are distributed homogeneously or

heterogeneously in the sarcolemma and T-tubules membrane. In the following I shall try to give you some information obtained in our studies on membrane transport in skeletal and heart muscle. The presentation will be concentrated on three major questions:

1./ Is it possible to separate the inward potassium movement to a sodium coupled active and an electrically coupled exchange process by ouabain and physostigmine?

2./ What sort of transport takes place across the T-tubular membrane?

3./ What distribution pattern of Na pumping sites can be found in the left and right ventricle of the rat heart?

Physostigmine- and ouabain-sensitive K influx in frog muscle

Cardiac glycosides have been widely used for the identification and determination of the active component of Na^+-K^+ transport. In muscle, however, the effects of compounds primarily affecting the passive fluxes have rarely been characterized in detail (Dahl-Hansen and Clausen, 1973).

The possibility of obtaining selective inhibition of the passive component of the K inward movement was suggested by the previous observations of Varga and Horowicz (1963), Kovács and Szabó (1972), who claimed that physostigmine inhibits potassium fluxes, and sodium influx in freshly dissected muscle.

Experiments were carried out on sartorius muscle of Rana esculenta. Muscles were incubated in radioactive Ringer for 30-60 min. At the end of exposition, the extra-cellular space was washed out by soaking muscle in an inactive solution at $4^{\circ}C$ for 5 min. Radioactivity was determined by counting aliquots of muscle extracts.

Fig. 1 demonstrates that ^{42}K uptake was linear with time. The average rate of K uptake was 7 $\mu mol \cdot g^{-1} \cdot hr^{-1}$. When muscles were preincubated with ouabain (0.5 mmol/l) and glycoside was also present during the radioactive exposition a significant 25 % reduction in the rate of ^{42}K uptake was obtained. Previously it has been shown that the effect of ouabain is a function of intracellular sodium concentration (Sjodin and Beaugé,

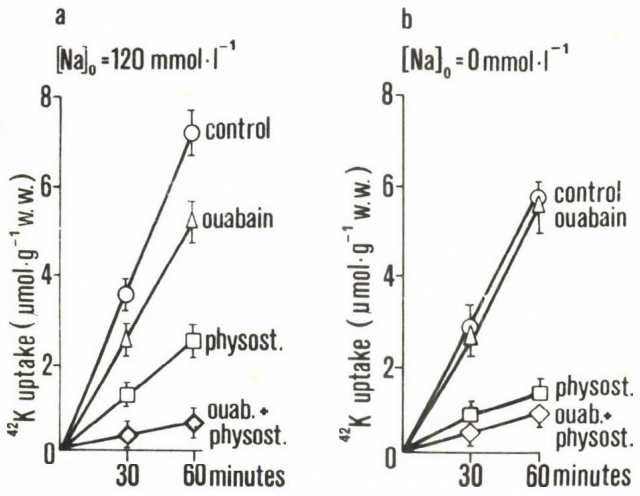

Fig. 1: Effects of 0.1 mmol/l ouabain and/or 1 mmol/l physostigmine on the K uptake in normal-sodium, and in low-sodium muscle

1968). Our experiments were performed in a paired muscle design. One member of the pair was incubated in normal Ringer with 120 mmol/l Na^+. The intracellular Na concentration of these muscles was about 12-13 mmol/kg i.f.w. In these muscles about 20-25 % of potassium influx was apparently ouabain-sensitive. When other pairs of muscles were preincubated in a Na-free, choline Ringer for 2 hr, their intracellular Na concentration fell down to about 6 mmol/kg i.f.w. In these low sodium muscles the potassium uptake was considerably lower; the average rate was 5.5 $\mu mol \cdot g^{-1} \cdot hr^{-1}$, and only a small fraction of the potassium influx proved to be ouabain-sensitive.

As compared to ouabain, physostigmine caused a more marked inhibition of ^{42}K uptake (Fig. 1). The inhibitory action of physostigmine was independent of intracellular Na^+ concentration since both in normal and low sodium muscles the application of physostigmine at a concentration of 1 mmol/l reduced the ^{42}K influx by about 70-75 %. When physostigmine and ouabain were added simultaneously to the preincubating and ^{42}K-Ringer solutions, about 90 % of K uptake was inhibited. Since ouabain and physostigmine have an additive action on the K uptake, our

results indicate that an ouabain-insensitive fraction of K influx was inhibited by physostigmine.

Sjodin (1971) reported that about 70-75 % of potassium movement in freshly dissected muscle is an electrically coupled K:K exchange, which is insensitive to cardiac glycosides. It seems reasonable to assume that physostigmine exerts its inhibitory action on this ouabain-insensitive K:K exchange process. This assumption is supported by the additive action of physostigmine and ouabain, the lack of any net change in the intrafibre potassium concentration in the presence of physostigmine and a decrease of ^{42}K efflux.

Cation transport across T-tubular membrane

Muscle fibres preincubated in hypertonic glycerol Ringer exhibit vacuolation of T-tubules after being exposed to normal Ringer. The glycerol procedure has frequently been used for the dissociation of excitation-contraction coupling. Henderson (1970) reported that the potassium uptake of frog sartorius muscle was reduced when T-tubules were disrupted by hypertonic glycerol treatment. Our findings also confirm Henderson's results, since glycerol treatment gave a lower uptake of ^{42}K, as compared to controls, at each time of incubation, although uptake remained linear with time (Fig. 2).

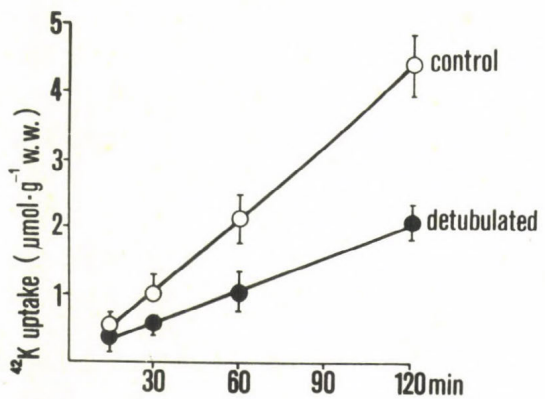

Fig. 2: ^{42}K uptake in control and detubulated companion muscles. Treated muscles were exposed to Ringer solution plus 400 mmol/l glycerol for 1 hr and returned to isotonic Ringer for 1 hr before uptake was measured

The effect of ouabain and physostigmine on the K influx was tested in normal and detubulated muscle pairs. Fig. 3 shows that disruption of T-tubules reduced the ^{42}K influx to the half of the control values. The reduction of ouabain insensitive fraction of K influx was marked in detubulated muscle but, as Fig. 3 reveals, the ouabain-sensitive K influx remained unchanged after glycerol treatment. On the other hand, it is noticeable that physostigmine-sensitive ^{42}K influx markedly decreased, without any significant change in physostigmine insensitive influx.

Results suggest that by far the greatest part of decrease in K influx caused by the disruption of T-tubules was due to a marked reduction of physostigmine sensitive pathways.

The effects of physostigmine on K efflux from normal and detubulated muscles were also compared. Fig. 4 shows that the efflux from glycerol treated muscle was less sensitive to physostigmine than it was from normal muscle. These results indicate that the inhibitory effect of physostigmine is greatly reduced by glycerol treatment. It seems reasonable to state that the physostigmine sensitive K:K exchange diffusion is located on the tubular membrane, and the ouabain-sensitive

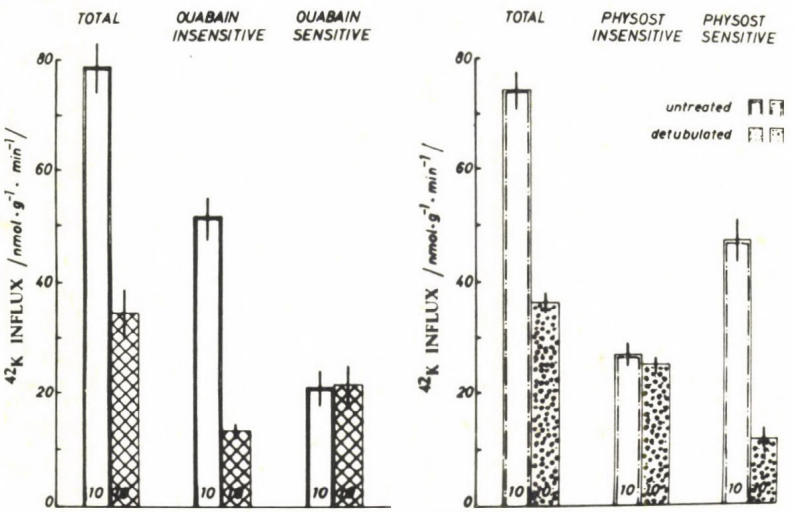

Fig. 3: Effect of ouabain and physostigmine on the K influx in normal and detubulated muscles

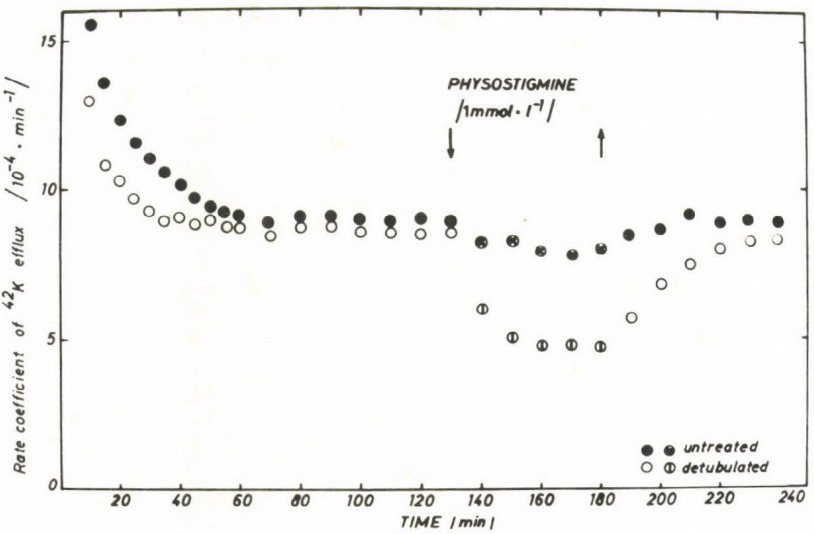

Fig. 4: Potassium efflux response to physostigmine (1 mmol/l). Filled circles from glycerol-treated muscle; open circles from untreated control muscle

Na:K exchange mediated by Na-K-ATPase can be located solely on the sarcolemma of the frog striated muscle. Our results are in good agreement with the findings of Venosa and Horowicz (1981), Narahara et al. (1979) who report that the density of ^{3}H-ouabain binding sites is considerably higher in the surface membrane, than in the T-tubular membrane.

Distribution of active pumping sites in rat ventricular muscle

The activity of sodium pump can be regulated by various factors. Overall sodium pump is determined by the number of active pumping sites and their turnover rates. Yamamoto et al. (1981) reported that the concentration of sodium pump is higher in ventricle than in atrial muscle of guinea-pig. In bovine heart, the Na-K-ATPase activity is lower in Purkinje fibres than in papillary muscle homogenate (Palfi et al., 1978). Myocardial ^{86}Rb uptake and ouabain binding are higher in newborn guinea-pigs and dogs than in the corresponding adult animals (Marsch et al., 1981).

The aim of our further experiments was to find out the regional differences or lack of differences in Na pumping and ouabain binding sites in the left and right ventricle of the rat. Three ventricular ring-preparations were made from the base to the apex of rat ventricle, and ^{42}K and ^{86}Rb uptake were measured in these preparations. At the end of the exposition in radioactive Tyrode solution, the extracellular space was washed out with cold, inactive solution, and the walls of the right and left ventricle were isolated, and their radioactivity was determined.

Fig. 5 shows the total, ouabain-sensitive and ouabain-insensitive K uptake into different regions of the right and left ventricle. Each fraction of K influx was considerably higher in the right ventricle than in the left one. Moreover, a remarkable regional difference was found in the K influx of the right ventricle. Ouabain-sensitive K influx was significantly lower in the middle part of the right ventricle than in the base and apex. Regional difference was also observed in the ouabain-insensitive K influx of the left ventricle. Although the differences between the various regions were less remarkable.

One possible explanation for the regional difference in K influx is the regional difference in sodium influx rate. Yamamoto et al. (1979), Akera et al. (1981) demonstrated that Na influx is a primary determining factor in the regulation of ouabain-sensitive ^{86}Rb influx. Therefore, the ouabain-sensitive ^{86}Rb influx can be considered as a specific indicator of sodium pump. The enhancement of specific Rb influx on the effect of either electrical stimulation or nonensine (Na ionophore) supports their assumption.

Therefore, ^{86}Rb influxes into the right and left ventricular regions were compared under rest and supramaximal electrical stimulation with 1 Hz frequency. Stimulation caused a marked rise in ^{86}Rb influx. The greatest enhancement was found in the apex of the right ventricle (Fig. 6).

Comparing the ouabain-sensitive to the ouabain-insensitive Rb influxes in stimulated left and right ventricular preparations we can state the following. Ouabain-sensitive Rb influx is about 2-3-fold higher in the right than in the left

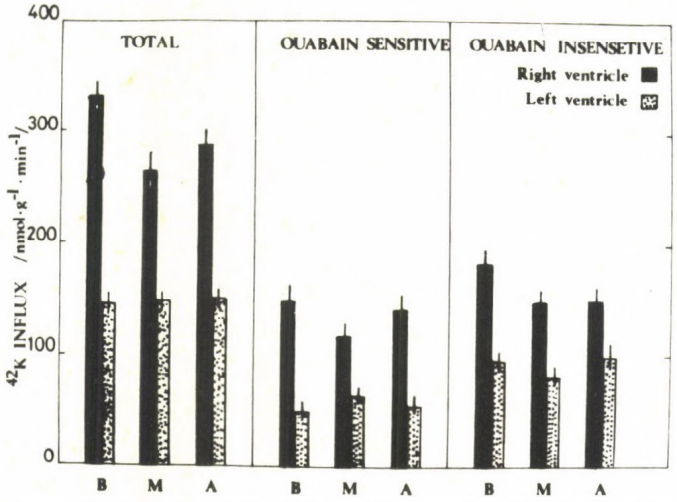

Fig. 5: Total, ouabain-sensitive, and ouabain-insensitive ^{42}K Uptake in different regions of right and left ventricles.

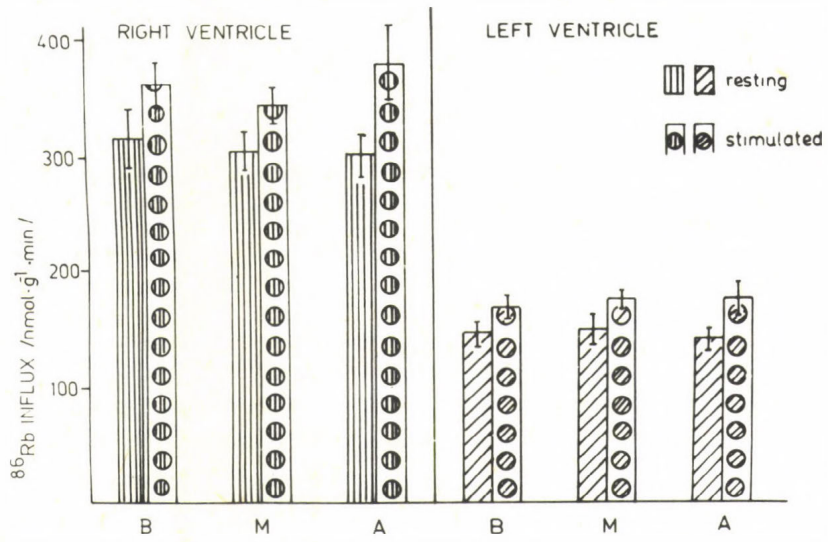

Fig. 6: Comparison of ^{86}Rb influx of different regions of rat ventricle under resting state and electrical pacing at 1 Hz

ventricle. The apical region of the right ventricle is bearing the highest ouabain-sensitive and lowest ouabain-insensitive influx. The same is true concerning the middle region of the left ventricle (Fig. 7).

Results may suggest regional differences in sodium influx, which in turn is able to induce a regional enhancement in the sodium pump activity. Recently Watanable et al. (1983) have reported that the action potential configuration in rat ventricle is heterogeneous. Action potentials were characterized in terms of amplitude, overshoot, duration at 25 % and 75 % repolarization (APD_{25}, APD_{75}). The shortest duration of repolarization was found in the right ventricle (APD_{75} = 21 ms). The action potential duration in the left ventricle is longer in the base (APD_{75} = 44 ms) than in the apex (APD_{75} = 36 ms), which in turn was significantly longer than those of right ventricle. The heterogeneity of the action potential may be due to relative differences in the accumulation of intracellular sodium ions. Subsequently the rise of cellular sodium may increase the outward current by stimulating the electrogenic sodium pump.

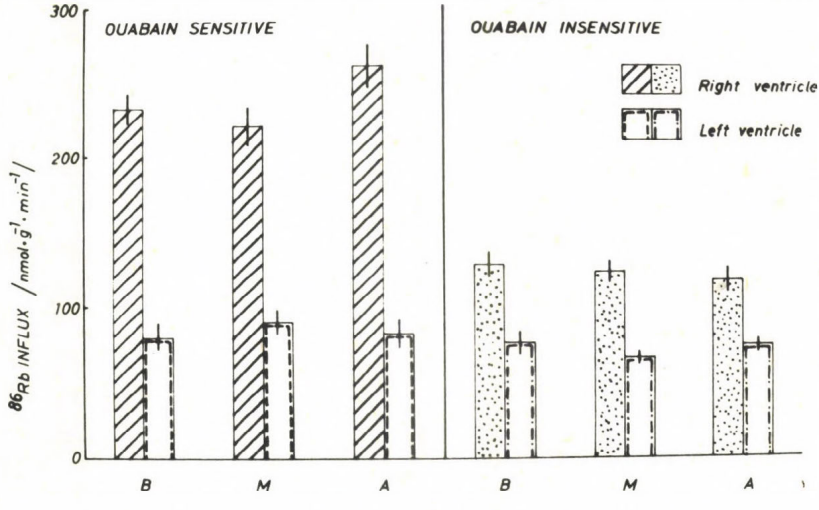

Fig. 7: Heterogeneity of ouabain-sensitive and insensitive Rb influx in right and left ventricle of the rat. Preparations were stimulated by supramaximal electrical pulses at a frequency of 1 Hz

It cannot be ruled out, however, that besides the regional difference in sodium influx, regional difference in the number of pumping sites may also affect the ^{86}Rb influx. To solve the question, it seemed necessary to examine the concentration of Na-K-ATPase and its affinity towards ouabain by determining the ATP dependent ^{3}H-ouabain binding.

Since the purification process inevitably causes a substantial loss of a fraction of the enzyme, and may also alter the affinity of the enzyme for ouabain, ^{3}H-ouabain binding was tested with the homogenates of the upper and middle parts from right and left ventricles, rather than purified enzyme preparations. Fig. 8 shows ATP dependent ^{3}H-ouabain binding in the upper and middle parts of right and left ventricles and their Scatchard plots. The number of binding sites, B_{max}, and dissociation constants, K_D, were estimated from the intercepts and slopes of the linear regression lines. The number of binding sites for ouabain in the base and middle portion of right ventricle was about 2-times higher compared to that of the left ventricle. On the other hand, the dissociation constant (recip-

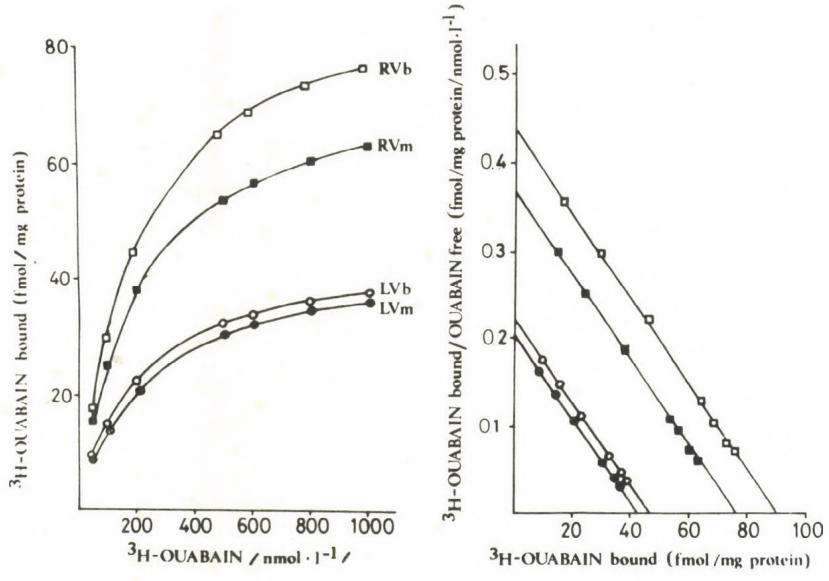

Fig. 8: Specific, ATP dependent ^{3}H-ouabain binding of homogenates from base and middle of right (RVb, RVm) and left ventricle (LVb, LVm). The binding data are also displayed as Scatchard plots.

rocal index of affinity) for ouabain was nearly the same in homogenates from left and right ventricles.

These results are in good agreement with the ouabain-sensitive Rb influx data, and indicate that the number of pumping sites is lower in the left than in right ventricle.

In summary, the various pharmacological compounds are useful means in the separation of the overall transport to different fractions in the excitable membrane. The distributions of cation transport pathways are different in the sarcolemmal and tubular membrane of skeletal muscle. The lack of sodium pump in tubular membrane may relate to the sodium dependent control of excitation-contraction coupling. The physiological role of the heterogeneous distribution of sodium pumping sites in the left and right ventricle is an open question yet, but it may have a significant influence on myocardial performance, especially in falling heart when cardiac glycosides are applied.

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DISCUSSION

IKEMOTO:

According to the recent report by Lattore et al. the T-tubule contains the Ca^{2+}-dependent K^{+} channels. Have you seen any difference of physostigmine effect depending upon the Ca^{2+} concentration?

KOVÁCS:

We have not sistematically investigated this possibility. In our experiments two concentrations of external Ca^{2+} were applied, namely Ca^{2+}_{e} was 1.8 $mmol \cdot l^{-1}$ in the normal Ringer and 5 $mmol \cdot l^{-1}$ in the 5-Ca-Mg-Ringer solution. But the degree of inhibition caused by physostigmine on K:K exchange was about the same in both solutions. It is likely, however, that the activation of the channels is brought about by the intracellular Ca^{2+}, while the extracellular one is ineffective.

TIGYI:

You showed functional (indirect) evidence of the different localizations of K pathways in various parts of muscle membrane. The use of ELMI radioautography could give you a direct evidence of pathway localizations. The method is available in our laboratory.

KOVÁCS:

This is a very interesting suggestion. I appreciate your kind offer, and I shall take the opportunity.

AKERMAN:

What is the effect of physostigmine on action potential? What concentration of physostigmine do you use?

KOVÁCS:

Physostigmine, in a mmolar concentration slows the rise as well as the fall of the spike. The spike duration is

also affected, it increases by about 90-95 % of the normal compared to the control's duration. Thus, physostigmine slows not only the rise of the spike, but it also prolongs the spike (Taylor et al., J. Gen. Physiol., 59:421, 1972; Szűcs et al., Acta Physiol. Hung., 62:1, 1983). In our experiments the concentration of physostigmine was 1 $mmol \cdot l^{-1}$.

SCHATZMANN:

Is the physostigmine sensitive K:K-exchange in skeletal muscle realized by a truly diffusional exchange mechanism or is it due to net movements across two separate pathways?

KOVÁCS:

We have reason to assume that the physostigmine sensitive K:K exchange is a truly diffusional exchange. Physostigmine caused the same degree of inhibition both in the efflux and influx, and it led to no net change in the intrafibre potassium concentration. On the other hand, Sjodin and Beaugé (J. Gen. Physiol., 52:389, 1968) reported that about 70-75 % of potassium movement in freshly dissected frog muscle is an electrically coupled K:K exchange, insensitive to cardiac glycosides. Our finding, that the average reduction in potassium influx brought about by the drug amounted to 70-75 % is in good agreement with Sjodin's data.

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ON THE ROLE OF PHOSPHATE IONS IN THE MEMBRANE POTENTIAL GENERATION OF MITOCHONDRIA

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It is known since the early nineteen-sixties that mitochondria are capable of accumulating large amounts of the divalent cations Ca^{2+} and Sr^{2+}. The recognition of the physiological importance of intracellular calcium movements placed this problem again in the focus of interest (Nicholls and Akerman, 1982).

The constituents of the mitochondrial inner membrane playing essential role in calcium transport are summarized in the scheme of Fig. 1. According to the chemiosmotic model of energy conservation (Mitchell, 1968) the proton-translocating systems of the respiratory chain and the low proton-permeability of the inner mitochondrial membrane give rise to transmembrane chemical (ΔpH) and electric ($\Delta\psi$) potential differences. The relative contribution of the two components has been shown to vary in a wide range. Their sum, the total proton electrochemical potential difference ($\Delta\mu_{H^+}$) is however kept constant by adequate stimulation or inhibition of the respiratory activity.

It is this inside negative membrane potential which allows the uptake of various cations against their concentration gradient into the matrix space. Ca^{2+}, Sr^{2+} and K^+ in the presence of valinomycin were shown to be accumulated under these conditions. However, the electric capacity of the inner membrane is so low that the transfer of negligible amounts (less than 1 nmol per mg) of cations would completely discharge the membrane potential. The respiratory chain is stimulated by the decreased $\Delta\mu_{H^+}$. The enhanced rate of H^+-ejection partially

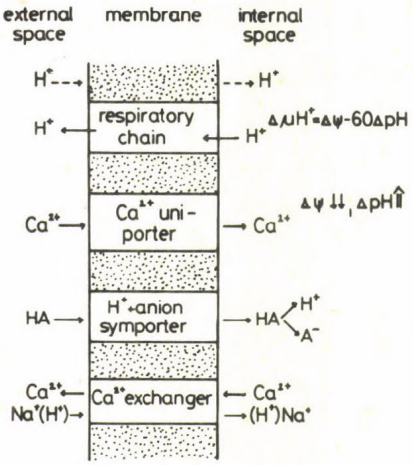

Fig. 1.
Model of the inner mitochondrial membrane illustrating the components participating in calcium transport processes

restores the depressed $\Delta\psi$ and augments the pH-difference, allowing further cation-uptake. However, this process is limited by the buffer capacity - 30-40 nmol per mg - of the intramitochondrial space and its exhaustion stops any further cation-accumulation. In this state the transmembrane pH difference is augmented by 1-1.5 units and the membrane potential is depressed by 60-90 mV to a final value of 70-100 mV.

Anion carriers represent the next important element in mitochondrial cation-accumulation. The phosphate, pyruvate, glutamate and beta-hydroxybutyrate translocators carry the respective anion in symport with protons through the hydrophobic phase, dissipating the pH gradient and stimulating the restoration of $\Delta\psi$. Similar effect is exerted by acetate, which diffuses in undissociated form through the lipid phase of the membrane. In the presence of these proton-donor anions large amounts of cations can be accumulated the process being accompanied by enhanced respiration and H^+-ejection.

Theoretically, net cation uptake should come to a standstillwhen the electrochemical equilibrium of the respective cation is reached. This is indeed the case when K^+-accumulation is studied in the presence of valinomycin. However, in the case of calcium, electrochemical equilibrium is never achieved in

well-coupled mitochondria. This observation started an intensive search for the second calcium transport pathway of mitochondria resulting in the discovery of a carrier exchanging internal calcium for external Na^+ in the heart and brain but for external H^+ in liver mitochondria (Crompton et al., 1978). Thus, the distribution of calcium between mitochondria and cytosol is determined by the activity of the two calcium transporting systems. A steady state between uptake through the uniporter and efflux via the exchange carrier is reached. For example a decrease of the extramitochondrial free calcium concentration might be the consequence of the stimulation of the uniporter or the inhibition of the exchange carrier.

It follows from the considerations explained above that the membrane potential could be depressed to a certain value without affecting calcium distribution. This was found indeed in experiments where $\Delta\psi$ was decreased by K^+ and valinomycin (Nicholls, 1978). However, a recent publication by Bernardi and Azzone (1982) has reported different results when $\Delta\psi$ was lowered by small amounts of uncouplers: they decreased $\Delta\psi$ and induced calcium-uptake. Phosphate had the opposite effect: it increased $\Delta\psi$ and at the same time induced calcium-release. As an explanation the cited authors suggested a new, outwardly directed, energy-dependent calcium pump in mitochondria.

These controversies prompted us to start a reinvestigation of the role of phosphate ions in the formation of the membrane potential, in the course of calcium movements and in the process of uncoupling.

CALCIUM ACCUMULATION IN THE PRESENCE OF VARIOUS PROTON-DONOR ANIONS

The membrane potential and the extramitochondrial free calcium concentration (expressed as the negative logarithm, pCa) were followed during calcium accumulation. The membrane potential was calculated on the basis of the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+), measured by an ion-selective electrode according to Kamo et al. (1979).

Changes of pCa were recorded by a calcium-sensitive electrode as detailed previously (Ligeti et al., 1981). Respiring mitochondria build up a membrane potential of 190-200 mV, which is further increased by the addition of 2 mM inorganic phosphate (Fig. 2A). The effect of the anion is prevented by mersalyl, an inhibitor of the phosphate carrier (Fig. 2B) but $\Delta\psi$ is promptly augmented upon the removal of mersalyl by mercaptoethanol (ME).

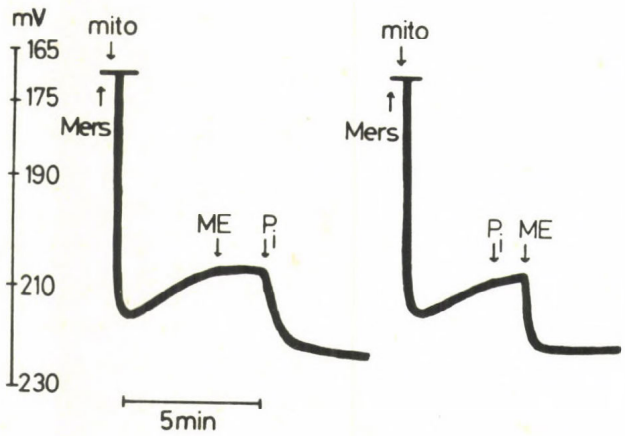

Fig. 2. Effect of phosphate on the membrane potential of respiring mitochondria. Trace of the TPP^+ electrode. The medium contained 245 mM sucrose, 10 mM Tris-HCl, 3 mM $MgCl_2$, 10 mM beta-hydroxybutyrate, 3,2 µM TPP^+ and 6 mg mitochondrial protein. The final pH was 7,0. mers.: 15 nmol mersalyl per mg protein, P_i: 2 mM Tris-phosphate, ME: 3 mM mercaptoethanol.

Thus phosphate ions influence the transmembrane potential difference only if they can enter the intramitochondrial space. Other proton-donor anions show similar tendencies but their effect is much weaker even if significantly higher concentrations are applied (not shown).

Calcium accumulation induces characteristic cyclic change of the membrane potential: a rapid drop followed by a slower restoration of $\Delta\psi$. Fig. 3 illustrates that in the presence of 0.5 mM phosphate these cycles could be repeated several times and the membrane potential always returned to the original value. In contrast to this, consecutive calcium pulses significantly lowered the steady state of $\Delta\psi$ if 5 mM beta-hydroxybutyrate served as accompanying anion. Glutamate and acetate behaved similarly to beta-hydroxybutyrate.

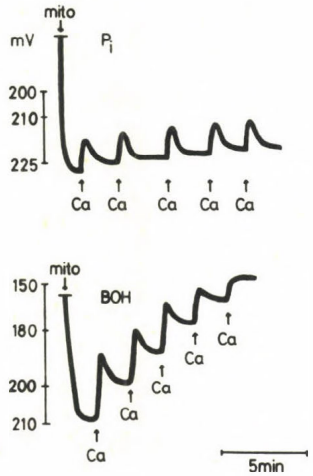

Fig. 3.
Effect of calcium on the mitochondrial membrane potential in the presence of 0.5 mM phosphate or 5 mM beta-hydroxybutyrate. Traces of the TPP^+ electrode. Experimental conditions were the same as in Fig. 2. Each Ca-pulse represents the addition of 17 nmol $CaCl_2$ per mg protein.

Recording of the extramitochondrial calcium concentration also revealed considerable differences between the effects of the various proton-donor anions. Mitochondria accumulate calcium ions from the external medium and a steady-state is reached between influx and efflux. This is shown by the stable electrode trace. A small pulse of calcium causes a transient increase in the extramitochondrial concentration, but in the presence of 0.5 mM phosphate pCa is rapidly returned to its original value (Fig. 4). As shown in the Figure, after four pulses mitochondria have accumulated 133 nmol calcium per mg protein and pCA decreased less than 0.15 unit. Different

results were obtained if phosphate was substituted by any other proton-donor anion: after each calcium-pulse steady-state was achieved only at a significantly lower pCa value. In the case of beta-hydroxybutyrate four calcium pulses (133 nmol per mg) decreased pCa by more than one unit (Fig. 4).

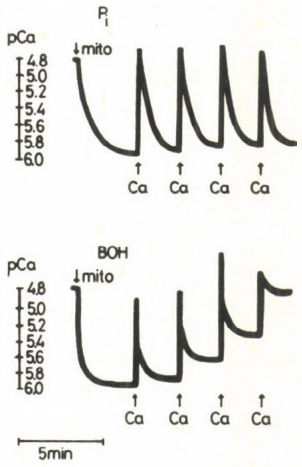

Fig. 4.
Effect of calcium on the steady-state pCa in the presence of 0.5 mM phosphate or 5 mM beta-hydroxybutyrate. Trace of the calcium electrode. Experimental conditions were the same as in Fig. 2, but TPP^+ has been omitted. Each Ca-pulse represents the addition of 33 nmol $CaCl_2$ per mg protein.

On the basis of the experiments of Lehninger (1974) and Harris (1978) the H^+-donor anions were generally regarded as being equally effective in supporting calcium-uptake into mitochondria. Our data point to a special role of phosphate ions in this process. The difference in the dissociation constants of the applied weak acids provides a possible explanation for the observed phenomenon. As the second pK-value of phosphate is around 7, so a large part of this anion is present in our experiments as $H_2PO_4^-$, the true substrate of the carrier (Fonyó et al., 1982). The other proton-donor anions - due to their lower pK-value - are present in the extramitochondrial space in fully dissociated form whereas only the undissociated weak acid can cross the hydrophobic barrier. Thus, in spite of the higher total concentration of the acetate, beta-hydroxybutyrate and glutamate, the amount of the

transported species is significantly lower than in the case of phosphate. The difference in the effects of the phosphate and the other proton-donor anions could also be explained by suggesting a direct interaction between the intramitochondrial phosphate and the proteins of the respiratory chain responsible for H^+-ejection. However, at the present stage of the experiments this remains only vague speculation.

EFFECT OF UNCOUPLING ON THE MEMBRANE POTENTIAL AND CALCIUM DISTRIBUTION

It was shown by Nicholls (1978) that above 130 mV the steady-state distribution of calcium is independent of the variations of the membrane potential. In contrast to this, Bernardi and Azzone (1982) found that alterations of $\Delta\psi$ could shift the steady-state pCa: net uptake was observed if a drop of $\Delta\psi$ was initiated by small amounts of uncouplers and, oppositely, enhancement of $\Delta\psi$ by the phosphate induced calcium-release. These observations prompted us to follow the effect of uncouplers on the membrane potential and on pCa in parallel experiments. Mitochondria were allowed to accumulate 13 nmol calcium per mg protein and to reach a steady-state. Small amounts of CCCP were then added consecutively. Fig. 5 shows that each dose of the uncoupler induced a definite fall of $\Delta\psi$ but the first three pulses did not influence pCa. Calcium efflux occurred only after the fourth addition, when the concentration of CCCP reached 8 nM. At this stage the membrane potential was decreased to approx. 185 mV. Fig. 6 summarizes the results obtained in the presence of phosphate. The anion itself increased $\Delta\psi$ by 20 mV, while it did not alter pCa. Subsequent additions of CCCP gradually reduced the membrane potential, but in this case six pulses of the uncoupler (equal to 12 nM) were needed to alter calcium distribution and shift pCa to a lower value. Net calcium release started again when $\Delta\psi$ was depressed approx. to 150 mV.

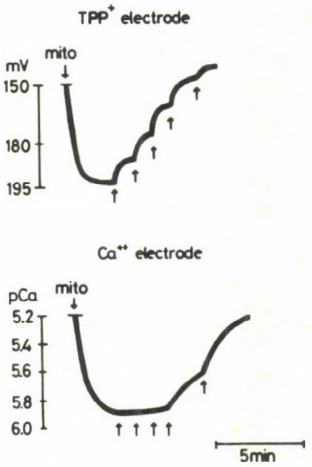

Fig. 5.
Effect of CCCP on the membrane potential (upper trace) and calcium movements (lower trace) in the absence of phosphate. Each arrow represents the addition of 2 nM CCCP.

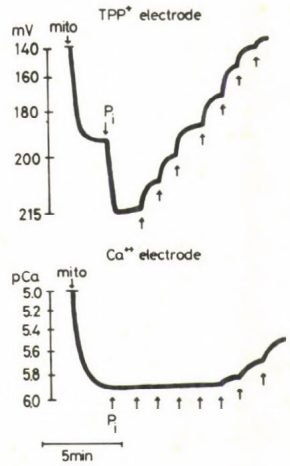

Fig. 6.
Effect of CCCP on the membrane potential (upper trace) and calcium movements (lower trace) in the presence of 1 mM phosphate. Each arrow represents the addition of 2 nM CCCP.

Thus, in contrast to the findings of Bernardi and Azzone (1982) we found that small variations of the membrane potential (either decrease by CCCP or increase by phosphate) have no effect on steady-state pCa. A large fall of $\Delta\psi$ (30-50 mV) induces calcium efflux and shifts the steady-state pCa to lower values. These results cast doubt on the membrane potential driven calcium efflux pathway hypothesized by these authors.

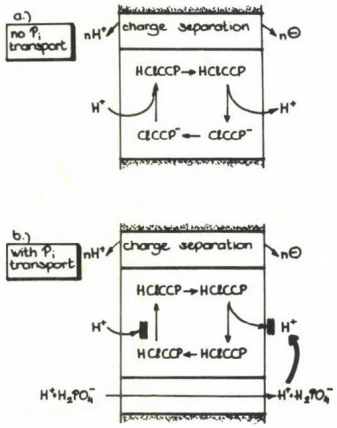

Fig. 7.
Model of the effect of CCCP and phosphate on the proton-permeability of the inner mitochondrial membrane

The effect of uncouplers is counteracted by phosphate, as in the presence of this anion more CCCP is needed to achieve a given value of $\Delta\psi$. The scheme of Fig. 7 explains this observation. The net translocation of protons by CCCP consists of the following elementary steps: 1. diffusion of the protonated form to the inner membrane/water interface, 2. dissociation of the weak acid and release of the proton, 3. return of the deprotonated base to the outer membrane/water interface, and 4. protonation of the base. An augmentation of the external phosphate concentration induces net phosphate uptake accompanied by an inward movement of protons and a decrease of the intramitochondrial pH. The more acidic internal milieu might interfere with the deprotonation of the uncoupler on the inner surface. As a result, a given amount of CCCP would be less effective, i.e., the same effect could be attained only by a higher concentration of the uncoupler.

CATION ACCUMULATION IN THE ABSENCE OF PROTON-DONOR ANIONS

As detailed in Fig. 1, the simultaneous uptake of proton-donor anions is a basic requirement for massive cation-accumulation into mitochondria. However, limited amounts of cations can be taken up also in the absence of these anions, too. It was

suggested that in this case the internal buffer capacity provides protons for the compensatory activity of the respiratory chain but the nature and ionic composition of the intramitochondrial buffers remained obscure.

We designed experiments where mitochondrial cation-uptake was measured at various levels of the internal phosphate content and the phosphate transport systems were blocked. Respiring mitochondria were either loaded by incubation in the presence of 2 mM phosphate or depleted by using up endogenous phosphate for ATP synthesis. Phosphate movements and ATP-hydrolysis were then stopped by mersalyl and oligomycin, resp. Cation-uptake was induced in this state by the addition of either calcium or the K^+-ionophore valinomycin, and the amount accumulated was assessed isotopically or by an ion-selective electrode.

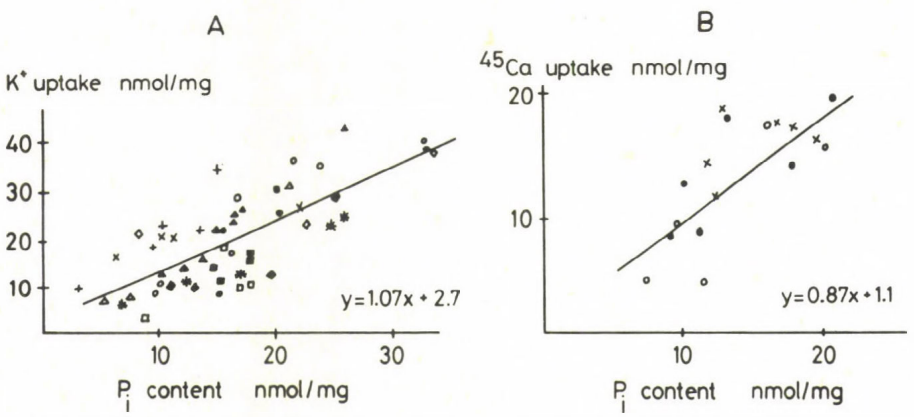

Fig. 8. Relationship between intramitochondrial phosphate content and the amount of accumulated K^+ (A) and ^{45}Ca (B)

Fig. 8 shows that in the case of both cations linear relationship was observed between the intramitochondrial phosphate content and the amount of the accumulated cation. The increase of the internal phosphate-level by one nmol per mg protein allowed the entry of one nmol K^+ or 0.87 nmol Ca^{2+} per mg protein. Thus, the same amount of endogenous phosphate supported

the uptake of more positive charges in the case of the divalent than in that of the monovalent cations. The difference could not be attributed either to phosphate-movement via the dicarboxylate carrier or to formation of bicarbonate from CO_2 (Harris, 1978) as both n-butylmalonate and Diamox were present in the experiments with calcium. The observed difference between mono- and divalent cations is in accordance with earlier reports (Fonyó and Ligeti, 1978) which showed that H^+-ejection accompanying calcium-uptake significantly exceeded H^+-pumping coupled to K^+-entry.

Our hypothesis is summarized in the scheme of Fig. 9. Phosphate is present in the matrix space in two different forms: as $H_2PO_4^-$ and HPO_4^{2-}. Dissociation of $H_2PO_4^-$ provides protons for the stimulated activity of the respiratory chain and the arising HPO_4^{2-} ions compensate for the entering positive charges.

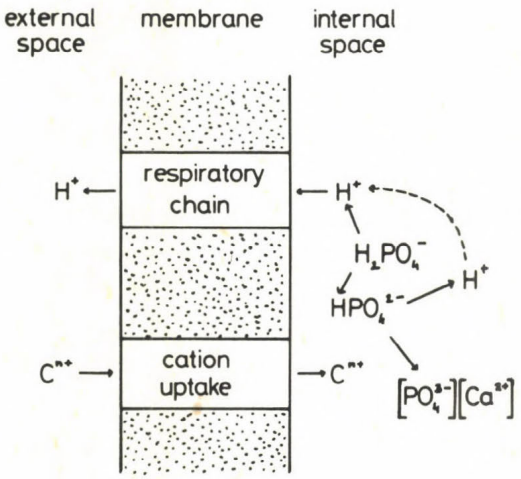

Fig. 9.
Model of the functions of intramitochondrial phosphate

In the case of the divalent cations the formation of insoluble precipitates with phosphate results in the liberation of additional protons, allowing the uptake of more positive charges than in the case of monovalent cations. Thus, phosphate ions present in the intramitochondrial space can be

regarded as a powerful buffer system of the matrix compartment and in addition they provide extra protons by formation of precipitates.

SUMMING UP

Our experiments have revealed a distinctive role of phosphate in the course of mitochondrial cation transport.

1./ Phosphate brings about the rapid dissipation of the transmembrane pH gradient and stimulates the charge separating activity of the respiratory chain. In this way phosphate interconverts ΔpH into $\Delta\psi$ and allows the complete restoration of the membrane potential after calcium accumulation. Dissipation of ΔpH even prevents the effect of low concentration of uncouplers.

2./ Phosphate forms insoluble salts with divalent cations. This process results in liberation of protons, providing substrate for the respiratory chain. On the other hand, precipitation of calcium keeps the intramitochondrial calcium concentration and consequently the rate of efflux through the exchange carrier at a constantly low level (Zoccarato et al., 1982).

3./ Phosphate, present in the matrix space is an essential component of the internal buffer system, serving as proton-supply for the proteins of the respiratory chain.

All these effects - keeping $\Delta\psi$ high, the rate of calcium efflux low and increasing the internal buffer capacity - favour rapid calcium uptake into the mitochondria in cases of an acute rise of the extramitochondrial concentration. The fact, that pCa was kept constant only in the presence of phosphate (Fig. 4) supports the idea that this anion represents an indispensable factor in the calcium buffering function of mitochondria.

ACKNOWLEDGEMENTS

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DISCUSSION

AKERMAN:

Can you explain any reason for the discrepancy between your and Dr. Bernardi's results.

LIGETI:

I can't explain the discrepancy. We tried to repeat Bernardi and Azzone's experiments but we could never get their results. There were some minor differences between the reaction media, for example we had no albumin present (what is the reason for the different CCCP concentrations applied) but I don't think this could give an explanation.

ERNSTER:

Did oligomycin have any influence on the effects of P_i observed in your experiments?

LIGETI:

No, we got the same results in the presence of oligomycin.

WOJTCZAK:

You can deplete mitochondria of endogenous phosphate by incubating them with hexokinase plus glucose. Have you examined the generation of membrane potential and ΔpH in such mitochondria?

LIGETI:

We didn't deplete mitochondria by glucose and hexokinase, we used only ADP. So I don't know whether mitochondria build up $\Delta\psi$ in the presence of HK + glucose.

AKERMAN:

Did you have ADP present in your system?

LIGETI:

ADP was present in the experiments on ^{45}Ca uptake in the absence of anions, but it was not present in the experiments with uncouplers.

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THE CRYSTALLIZATION OF Ca^{2+} TRANSPORT ATPase IN SARCOPLASMIC RETICULUM

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SUMMARY

1. Vanadate and lanthanide ions induce the formation of Ca^{2+}-ATPase crystals in sarcoplasmic reticulum which are assumed to reflect the E_2 and E_1 conformations of the enzyme, respectively.

2. Similar crystals were obtained after vanadate treatment of sarcoplasmic reticulum vesicles isolated from fast and slow skeletal and cardiac muscle of rats and rabbits and from normal and genetically dystrophic muscles of man, chicken and mouse. This is in accord with the biochemical similarity of Ca pumps in different muscle types of several species.

3. Treatments that are known to influence the conformational equilibrium of Ca^{2+}-ATPase (Ca^{2+}, ATP, inorganic phosphate, pH, temperature, ionic strength, membrane potential, proteolysis) interfere with the crystallization process.

4. Low dose electron microscopy and image reconstruction established ATPase dimers as the structural units of the crystals and confirmed the existence of structural domains within ATPase molecules, that can also be seen by high resolution electron microscopy of negatively stained or deep-etched and rotary shadowed preparations. Refinement of the image reconstruction, already in progress, is expected to yield the three-dimensional structure of the E_2 form of Ca^{2+}-ATPase at 25 Å resolution.

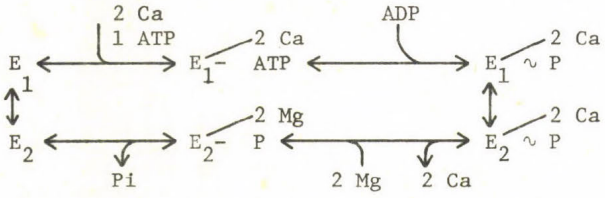
5. Experiments are in progress for the production of three-dimensional crystals of Ca^{2+}-ATPase that may carry the refinement of the structure to atomic detail.

INTRODUCTION

The Ca^{2+} transport ATPase of sarcoplasmic reticulum is a lipoprotein of about 110,000 molecular weight, that constitutes 60-80 percent of the protein content of sarcoplasmic reticulum vesicles isolated from fast-twitch skeletal muscle (Martonosi, 1983). The purified Ca^{2+} transport ATPase reconstituted into phospholipid vesicles actively transports Ca^{2+} with the hydrolysis of ATP. The ATP dependent transport of Ca^{2+} by sarcoplasmic reticulum involves the following reaction steps (Scheme 1).

1. Interaction of Ca^{2+} with the Ca^{2+}-ATPase in the E_1 conformation.
2. Phosphorylation of the enzyme by ATP followed by conversion of the E_1 into the E_2 enzyme form.

3. Sequential release of Ca^{2+} and inorganic phosphate from the E_2 conformation.



Scheme 1. Elementary steps of Ca^{2+} transport and ATP hydrolysis. The enzyme alternates between two conformations (E_1 and E_2) during the Ca^{2+} transport cycle. The E_1 conformation is characterized by high affinity for Ca and ATP and low affinity for inorganic phosphate (Pi) and Mg^{2+}. The E_2 conformation has low Ca^{2+} affinity but relatively high affinity for Pi and Mg^{2+}.

The proposed alternation between two distinct conformations of the Ca^{2+}-ATPase during the transport cycle is supported by changes in the affinity of the enzyme for Ca^{2+}, Mg^{2+} and ADP (Martonosi, 1982c), the differences in the reactivity of protein functional groups (Martonosi and Beeler, 1983) and the responses of fluorescence (Dupont et al., 1982) and electron spin resonance probes (Inesi et al., 1982) at predicted stages in the Ca^{2+} transport process.

Electron microscope (Martonosi, 1968) and x-ray diffraction data (Worthington and Liu, 1973; Dupont et al., 1973; Dupont and Hasselbach, 1973; Herbette et al., 1977) indicate an asymmetric disposition of the Ca^{2+}-ATPase in the membrane, with major portions of the polypeptide exposed on the cytoplasmic surface, connected by short hydrophobic segments that are immersed in the lipid bilayer (MacLennan and Reithmeier, 1982). The hydrophilic portion of the molecule can be visualized on the cytoplasmic surface by negative staining in the form of 4.0 nm diameter particles with an average density of $\simeq 20{,}000/\mu^2$ (Jilka et al., 1975); this is close to the estimated concentration of Ca^{2+}-ATPase polypeptide chains in the membrane. The intramembranous regions of the molecule can be visualized by freeze-etch electron microscopy as 8.5 nm diameter particles that are about 10 times more numerous on the cytoplasmic than on the luminal fracture face (Deamer and Baskin, 1969). The combined density of 8.5 nm intramembranous particles ($\simeq 5000/\mu^2$) is less than the density of 40 Å surface particles ($\simeq 20{,}000$), although both sets of particles are clearly associated with the Ca^{2+}-ATPase. These observations suggested that the ATPase molecules associate in the membrane to form oligomers (Jilka et al., 1975). The existence of oligomers is also supported by ultracentrifuge, exclusion chromatography and fluorescence energy transfer studies (Møller et al., 1982; Martonosi and Beeler, 1983).

Compared with the impressive advances in our knowledge on the composition of sarcoplasmic reticulum and on the kinetic mechanism of ATP dependent active Ca^{2+} transport the structural changes associated with the operation of the Ca^{2+} pump are less clearly defined. It is generally assumed that energization of the Ca^{2+} pump by phosphorylation with ATP creates a transmembrane channel for the active accumulation of calcium, but we can only speculate about the conformational requirements and the chemical mechanism of the process.

We now report observations on the structure of Ca^{2+}-ATPase membrane crystals induced by exposure of native sarcoplasmic reticulum vesicles to two types of Ca^{2+} transport inhibitors that may provide structural clues to the mechanism of Ca^{2+} transport.

1. One of these inhibitors is Na_3VO_4, an analog of inorganic phosphate, that stabilizes the E_2 conformation of the enzyme by interaction with the phosphate binding site of the Ca^{2+}-ATPase. Therefore the Ca^{2+}-ATPase crystals induced by Na_3VO_4 are expected to reflect the geometry of ATPase-ATPase interactions characteristic of the E_2 enzyme conformation (Dux and Martonosi, 1983a).

2. The second class of inhibitors are lanthanides. Gd^{3+} and La^{3+} are Ca^{2+} analogs which are presumed to inhibit Ca^{2+} transport by interaction with the high affinity Ca^{2+} binding site of Ca^{2+}-ATPase in the E_1 conformation (Grisham, 1982).

In both systems crystallization of the enzyme requires inhibition of the Ca^{2+} modulated ATPase activity. We assume that the two crystal forms represent the conformation of the Ca^{2+} pump at the beginning and at the end of the transport cycle. Therefore analysis of the corresponding structures may provide information about the physical basis of Ca^{2+} translocation.

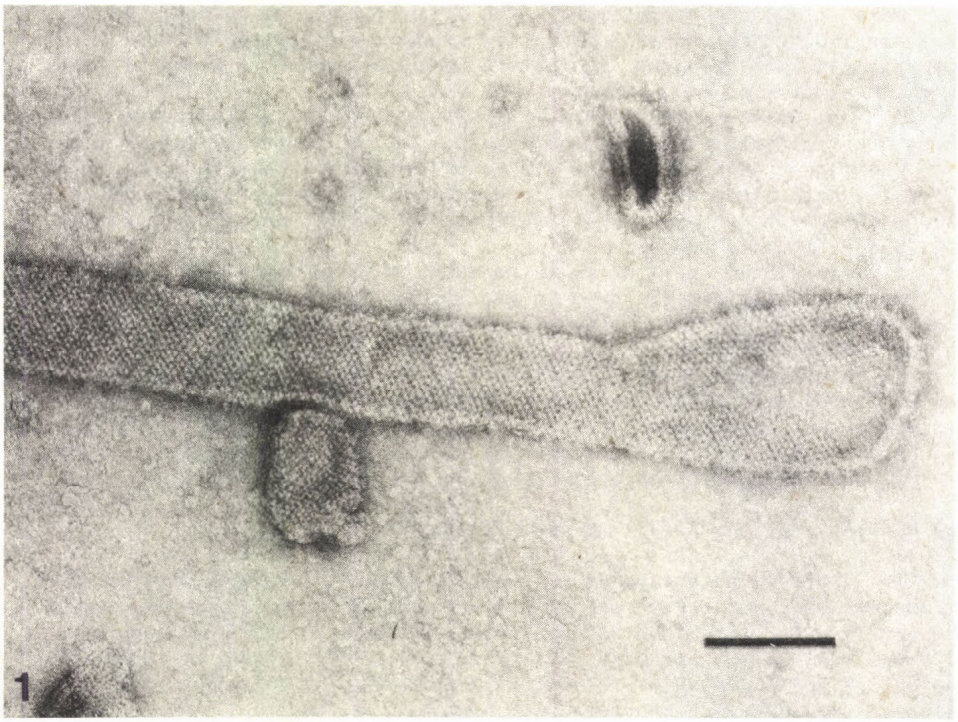
THE CRYSTALLIZATION OF Ca^{2+}-ATPase WITH Na_3VO_4.

Two-dimensional arrays of Ca^{2+}-ATPase crystals develop upon treatment of sarcoplasmic reticulum vesicles or reconstituted membranes containing the purified Ca^{2+} transport ATPase with 5 mM Na_3VO_4 at 2°C in a medium of 0.1 M KCl, 10 mM imidazole pH 7.4 and 0.5 mM EGTA (Fig. 1). Unphysiological pH (pH 5.0 or 9.0), elevated temperature (37°C), and high ionic strength (0.6 M KCl), interfere with crystallization.

Vesicles with extensive crystalline arrays assume elongated tubular shapes with average diameters of 50.0 - 70.0 nm, suggesting that the crystalline arrays are best accommodated by a cylindrical surface contour. The lattice lines are oriented diagonally at about 45-60° to the long axis of the tubules, and superimposition of the front and rear images of the flattened cylinders creates a diamond pattern of varied regularity (Fig. 1). At the end regions of the cylinders and in spherical vesicles the lattice is either absent or irregular. In regular arrays the rows of negatively stained particles are arranged in pairs forming dimer chains that are separated from neighboring chains by wider bands of negative stain. As a result the distance of separation between rows of particles alternates (Fig. 1). Occasionally the helical lattice unwinds at the end of the tubules (Fig. 2) revealing chains of ATPase dimers separated from neighboring chains by variable distances.

Interaction between ATPase molecules within the chains appears to be stronger than between them suggesting that the crystalline lattice develops in the following three stages (Fig. 3).

1. Formation of ATPase dimers.
2. Linear polymerization of ATPase dimers into tetramers and longer dimer chains by a process reminiscent of fibrous condensation (Oosawa and Asakura, 1975).
3. Lateral association of dimer chains into a two-dimensional right-handed helical lattice that surrounds the cylinders.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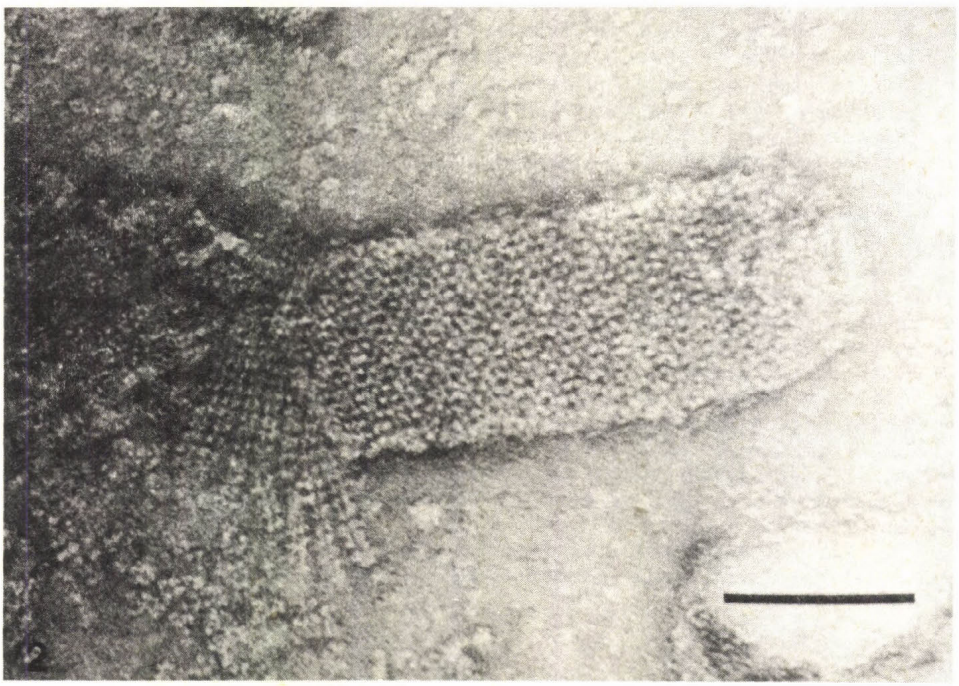
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Different types of bonds appear to be involved in the association of monomers into dimers (bond A), of dimers into dimer chains of diverse length (bonds B,C), and of dimer chains into extended two-dimensional lattice (bond D). The existence of monomers, dimers and tetramers of Ca^{2+}-ATPase was observed in native membranes and in the presence of detergents by ultracentrifugation, exclusion chromatography, target inactivation, fluorescence energy transfer and freeze-etch electron microscopy (Møller et al., 1982; Martonosi and Beeler, 1983). Long dimer-chains and extended crystalline arrays are only observed after treatment of sarcoplasmic reticulum with Na_3VO_4, which stabilizes the E_2 enzyme conformation, or after treatment with lanthanides (see below) that stabilize the E_1 conformation.

Fig. 1. Vanadate-induced Ca^{2+}-ATPase crystals in sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were incubated in 0.1 M KCl, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA and 5 mM Na_3VO_4 for 48 hours at 2°C. $GdCl_3$ (10^{-6} M) was added for an additional 48 hours followed by negative staining with uranyl acetate. Gadolinium (10^{-6} M) had no detectable effect on the Ca^{2+}-ATPase crystals preformed in the presence of vanadate. Bar: 100 nm.

Fig. 2. Separation of Ca^{2+}-ATPase "chains" in a disrupted vanadate-treated sarcoplasmic reticulum vesicle. Sarcoplasmic reticulum vesicles were treated with Na_3VO_4 for 48 hours, as described in Fig. 1, except that $GdCl_3$ was not added. At the lower end of the long crystalline tubule Ca^{2+}-ATPase "chains" are seen unwinding from a crystalline array, which consists of rows of Ca^{2+}-ATPase dimers. The width of the flattened crystalline tubule is about 1000 Å. The diagonal lattice arises from superimposition of images from the front and rear surfaces of the collapsed cylinder. Bar: 100 nm.

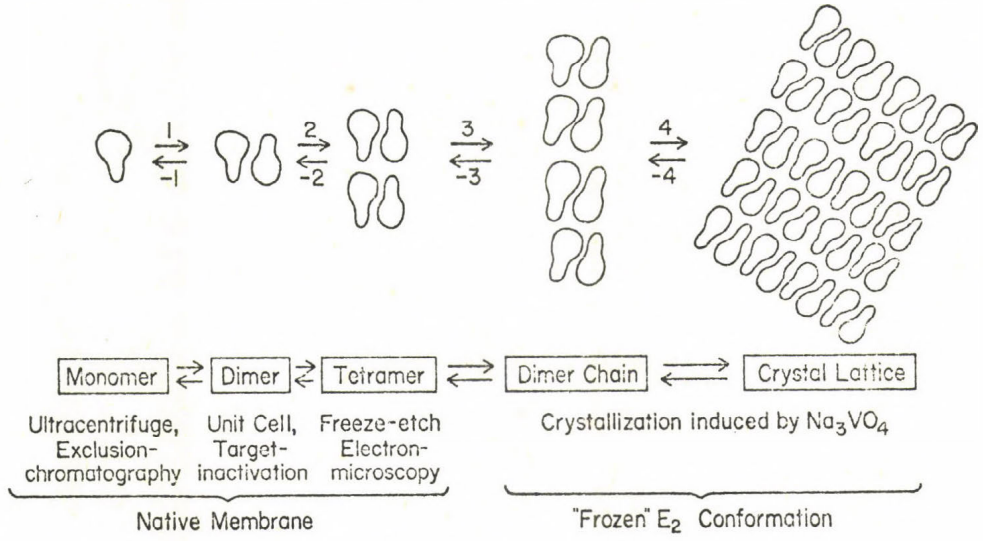


Fig. 3. Hypothetical scheme of ATPase-ATPase interactions.

Optical diffraction studies on vanadate-induced Ca^{2+}-ATPase crystals.

Digital Fourier transforms of the images of flattened tubules yielded lattice constants of a = 66 Å, b = 114 Å and γ = 78°. (Fig. 4).

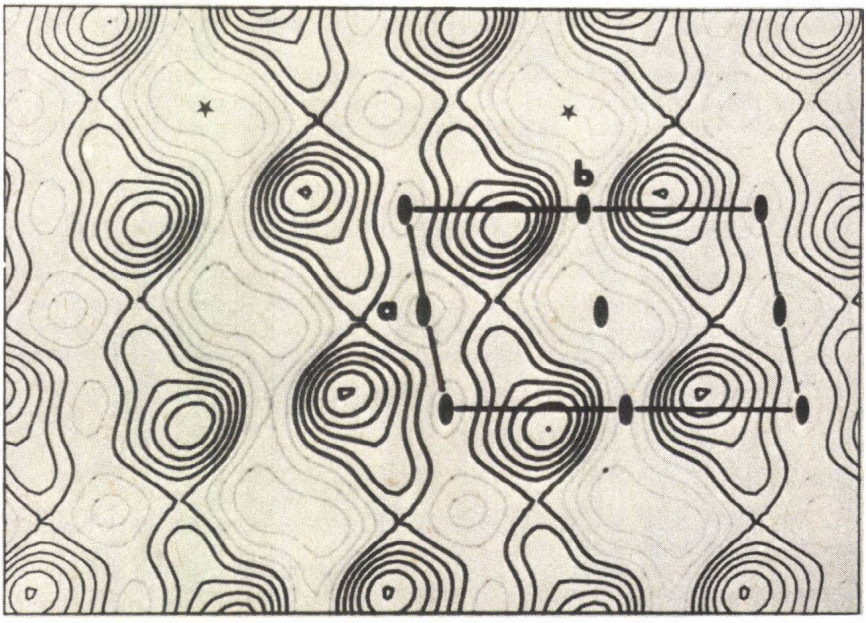


Fig. 4. Low-dose electron density map of vanadate-induced Ca^{2+}-ATPase crystals from rabbit sarcoplasmic reticulum, negatively stained with uranyl acetate.

The unit cell, its symmetry elements and the cell axes are identified. A pair of ATPase molecules constituting a dimer is encircled. The dimers form long chains that are wound around the tubules in a right-handed helix separated from neighboring dimer chains by wider bands of negative stain (*) (from Taylor et al., 1983, in preparation).

The average structure calculated by Fourier synthesis is consistent with ATPase dimers in which the two Ca^{2+}-ATPase molecules are separated by a center to center distance of about 64 Å (Taylor et al., 1983 in preparation).

A substructure of ATPase molecules is discernible in computer reconstructed images from averaged Fourier transforms calculated from low-dose electron micrographs (Taylor et al., 1983, in preparation). A similar substructure is also evident in ATPase molecules in regions of the surface lattice visualized by one sided staining with uranyl acetate, which show pairs of pear-shaped ATPase profiles with a stain filled region (channel?) enclosed between them (Fig. 5).

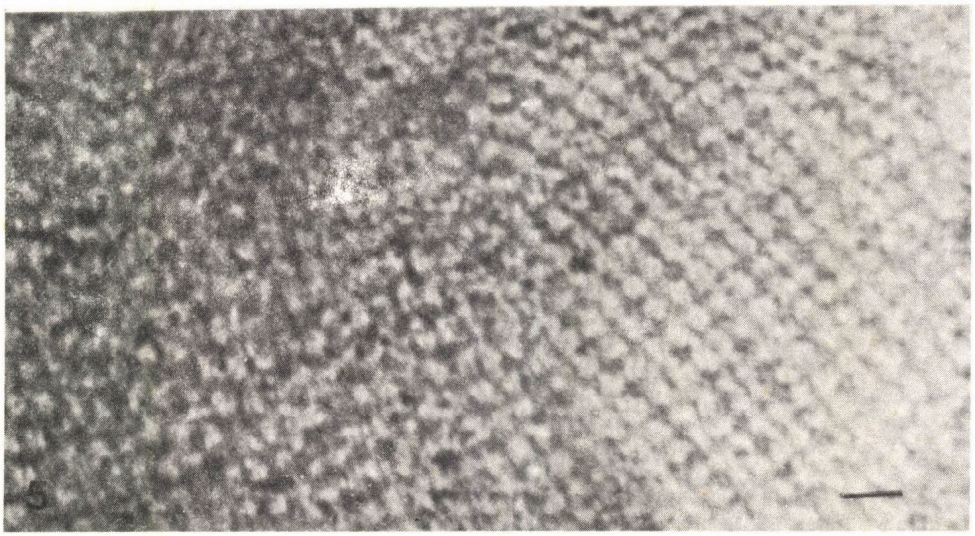


Fig. 5. High magnification picture of a disrupted sarcoplasmic reticulum vesicle showing the regular arrays of vanadate-induced Ca^{2+}-ATPase crystals.

Occasional lysis of vesicles during negative staining yields single sheets of Ca^{2+}-ATPase crystals adhering to the film. 7 ribbons are seen separated by wider bands of negative stain. Some ATPase particles are resolved into two distinct domains. Bar: 10 nm.

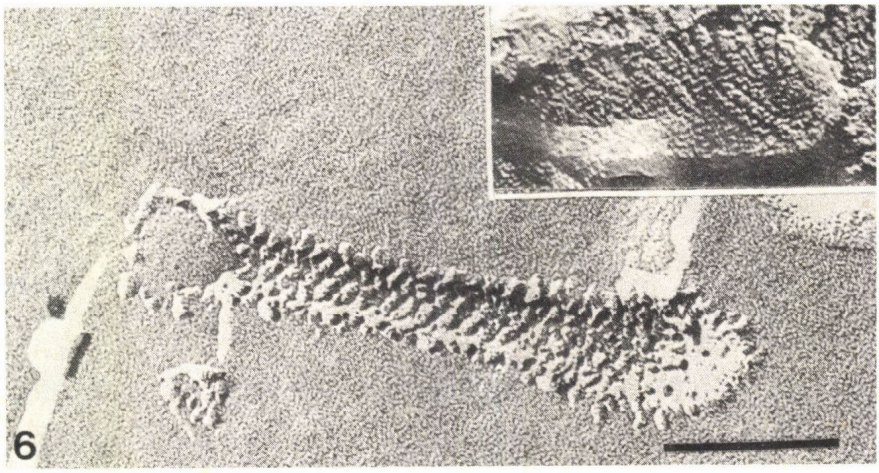


Fig. 6. Freeze-fracture replicas of sarcoplasmic reticulum vesicles treated with 5 mM Na_3VO_4.

Most vesicles have elongated into tubules 60-80 nm in diameter. The P-face of the tubules shows parallel ridges oriented at an angle of 50-60° to the long axis of the tubule. Bar: 100 nm. Insert: surface structure of a tubule after deep-etching and rotary shadowing (from Peracchia et al., 1983).

Freeze-etch electron microscopy of vanadate-induced Ca^{2+}-ATPase crystals.

In membranes containing Ca^{2+}-ATPase crystals the 8.5 nm intramembranous particles on the P-face are regularly organized into parallel ridges, that are coiled around the tubules in right-handed helices oriented at 50-60° angle to the long axis of the tubules (Fig. 6). The particles repeat along the rows at 5.5 nm and the rows repeat at 10.5 - 11.0 nm.

Deep etching and rotary shadowing reveal oblique crests on the protoplasmic surface consisting of particles 8.5 x 5.5 nm in size which represent dimers (Peracchia et al., 1983). The dimers are frequently resolved into monomers; within the monomers occasionally two distinct structural domains are seen, which correspond to those observed by negative staining (Fig. 5) and image reconstruction (Fig. 4).

On the convex (E) face of the tubules parallel rows of pits are visible (not shown), that are complementary images of the ridges (Peracchia et al., 1983). Since pits are not observed on the convex faces of sarcoplasmic reticulum vesicles in the absence of Na_3VO_4, we suggest that either the induction of the E_2 conformation or the subsequent crystallization of the Ca^{2+}-ATPase may be associated with deeper immersion of the polypeptide into the lipid bilayer, as shown schematically in Fig. 7.

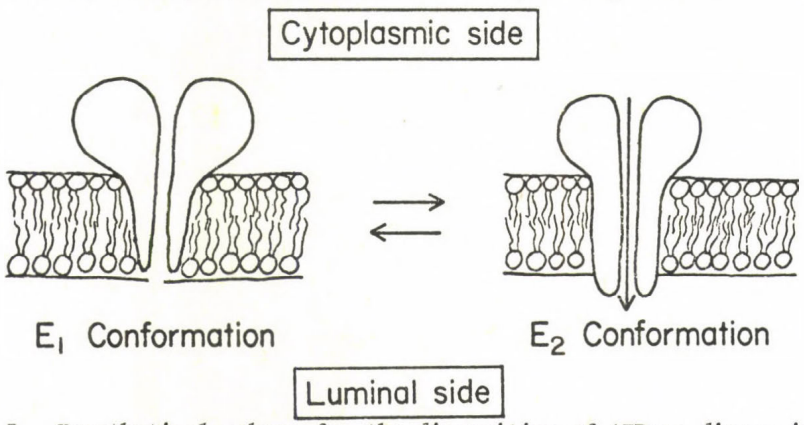


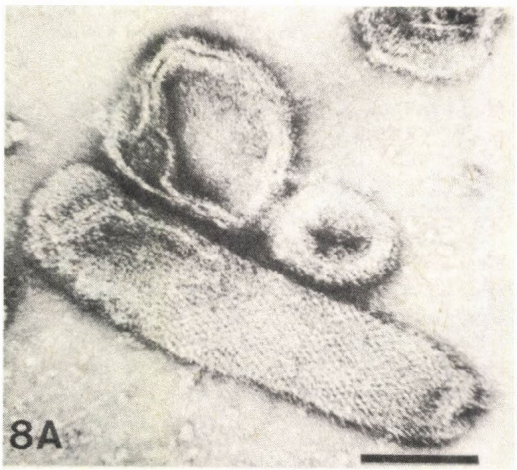
Fig. 7. Hypothetical scheme for the disposition of ATPase dimers in the membrane.

This hypothesis is consistent with the fact that Ca^{2+} translocation across the membrane occurs during conversion of the enzyme from the E_1 into the E_2 conformation.

CRYSTALLIZATION OF THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase INDUCED BY Gd^{3+} AND La^{3+} IONS.

Gd^{3+} binds at the Ca^{2+} binding site of the Ca^{2+}-ATPase in the E_1 conformation with an affinity about 10 times greater than that of Ca^{2+} (Grisham, 1982). At 10^{-6} M concentration which is expected to saturate the high affinity Ca^{2+} binding sites, $GdCl_3$ causes the crystallization of Ca^{2+}-ATPase in about 20% of the vesicles within 48 hours (Fig. 8, Dux and Martonosi, 1983g). The Gd^{3+} induced crystalline arrays form a diagonal lattice on the surface of tubules, but the contours of individual ATPase molecules are not as clearly defined as in vanadate-induced ATPase crystals. These observations lead to the impression that the ATPase particles project less prominently out of the bilayer in Gd^{3+} than in vanadate-induced crystals.

This may reflect either a different disposition of ATPase molecules in the bilayer, or the presence of accessory proteins (calsequestrin, Ca binding protein, etc.) on the vesicle surface in Gd^{3+} induced crystals; these proteins are removed by EGTA during Na_3VO_4 induced crystallization. La^{3+} (10^{-6} M) also caused crystallization of the Ca^{2+}-ATPase (Fig. 9); the Gd^{3+} and La^{3+} induced crystals were similar (Dux and Martonosi, 1983g).



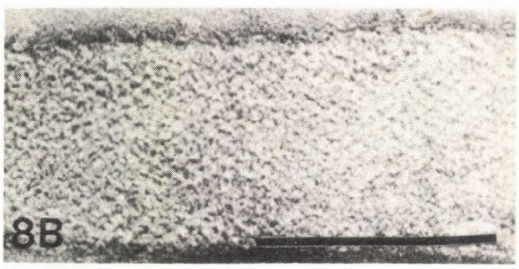


Fig. 8. Ca^{2+}-ATPase crystals induced by $GdCl_3$.

A. Sarcoplasmic reticulum vesicles were incubated in 0.1 M KCl, 10 mM imidazole pH 7.2, 5.0 mM $MgCl_2$ and 10^{-6} M $GdCl_3$ at 2°C for 48 hours, and samples were taken for negative staining with 1% uranyl acetate. Bar: 100 nm.

B. A portion of the vesicles seen in Fig. 8A at higher magnification. Bar: 100 nm.

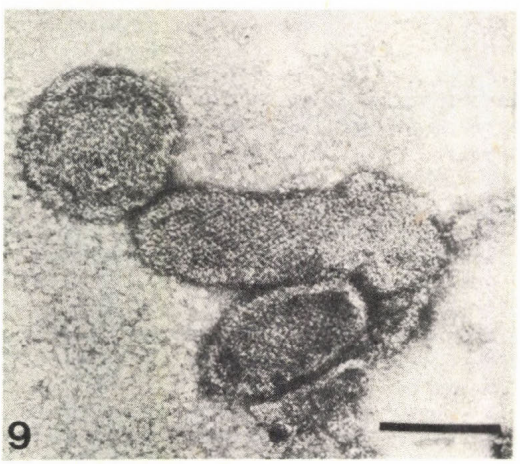


Fig. 9. Ca^{2+}-ATPase crystals induced by $LaCl_3$.

Sarcoplasmic reticulum vesicles were incubated in 0.1 M KCl, 10 mM imidazole pH 7.2, 5.0 mM $MgCl_2$ in the presence of 10^{-6} M $LaCl_3$ at 2°C for 5 days. Samples were stained with 1% uranyl acetate. Bar: 100 nm.

The optimum concentration of $GdCl_3$ and $LaCl_3$ for crystallization was about 10^{-6} M, that is barely sufficient to saturate the high affinity Ca^{2+} binding sites of Ca^{2+}-ATPase. At higher concentrations of either Gd^{3+} or La^{3+} the frequency of crystalline vesicles sharply declined presumably due to charge repulsions.

The crystallization of Ca^{2+}-ATPase by lanthanides raises the question, why such effect is not observed with Ca^{2+}? We assume that lanthanides are transported by the Ca^{2+} pump so slowly that in the presence of 10^{-6} M Gd^{3+} or La^{3+} essentially all pump molecules are frozen in the E_1 conformation, which promotes crystallization. Under similar conditions with 10^{-5} - 10^{-6} M $CaCl_2$ the rapid cyclic changes in enzyme conformation connected with Ca^{2+} transport inhibit crystallization.

In conclusion the studies described above suggest that the Ca^{2+}-ATPase

can be crystallized with lanthanides presumably in the E_1 conformation and with vanadate in the E_2 conformation. The E_1 and E_2 conformations represent the structure of the pump at the beginning and at the end of the Ca^{2+} transport cycle. Therefore three-dimensional reconstruction of these structures is expected to yield information about the conformation change of the enzyme related to Ca^{2+} translocation. These studies are in progress.

CONDITIONS AFFECTING THE FORMATION OF Ca^{2+}-ATPase CRYSTALS IN THE PRESENCE OF Na_3VO_4.

The effect of Ca^{2+}. Ca^{2+} in a concentration sufficient to saturate the high affinity Ca^{2+} binding site of the Ca^{2+}-ATPase (1-10 μM) prevents the formation of Ca^{2+}-ATPase crystals and "cracks" the crystals that were formed previously in the presence of 0.5 mM EGTA and 5 mM Na_3VO_4 (Dux and Martonosi, 1983a,b). Ca^{2+} in low concentration also protects the Ca^{2+} transport ATPase activity against inhibition by Na_3VO_4 (O'Neil et al., 1979; Pick, 1982; Pick and Karlish, 1982). Vanadate is an analogue of inorganic phosphate and is assumed to bind to the low affinity phosphate binding site of the enzyme, which is exposed only in the absence of Ca^{2+}; as a result the stable E_2-vanadate form of the enzyme accumulates.

The formation of two-dimensional crystals requires that most of the enzyme molecules assume the E_2 conformation; the inhibition of crystallization by Ca^{2+} may be attributed to stabilization of the E_1 enzyme form.

The effect of nucleoside phosphates. The formation of Ca^{2+}-ATPase crystals is inhibited by ATP (5 mM) but not by ADP, AMP, 5'adenylylimidodiphosphate (AMPPNP) or adenylyl methylenediphosphonate (AMPPCP) (Dux and Martonosi, 1983b). We did not observe any effect on the crystal structure when 5 mM ATP, ADP, AMPPNP, or AMPPCP were added to microsomes previously crystallized with vanadate in the absence of nucleotides. These observations suggest that binding of ATP to the enzyme interferes with crystallization presumably by changing the structure of the protein; once crystals are formed, ATP cannot disrupt the crystal lattice either because the ATP binding site becomes inaccessible or because the E_2 conformation of the enzyme is stabilized by the crystal structure against disruption.

The effect of inorganic phosphate. It is plausible to assume that vanadate inhibits the Ca^{2+}-ATPase and promotes its crystallization by forming an inactive E_2-vanadate complex at the inorganic phosphate binding site. In fact inorganic phosphate (1 mM, pH 6.0) mimics the effect of vanadate and promotes the crystallization of Ca^{2+}-ATPase in a Ca-free medium (Dux and Martonosi, 1983b). The crystal form obtained with inorganic phosphate was usually less regular and different from the vanadate-induced Ca^{2+}-ATPase crystals. Crystals were not produced by 1 mM inorganic phosphate at pH 7.0, where the affinity of Pi for the enzyme is low.

Effect of membrane potential on the formation of Ca^{2+}-ATPase crystals in the presence of Na_3VO_4. The rate of vanadate-induced crystallization is increased more than ten-fold by inside positive membrane potential generated by substitution of K-glutamate for choline chloride or K-methanesulfonate + valinomycin for Na-methanesulfonate in the incubation medium (Table 1; Dux and Martonosi, 1983c). Negative potential caused the transient disruption of preformed Ca^{2+}-ATPase crystals followed by slow reappearance of the lattice after the potential was dissipated (Dux and Martonosi, 1983c). Using oxonol VI and 3,3'diethyl-2,2'thiadicarbocyanine (Di-S-C_2(5)) to monitor inside positive and inside negative membrane potentials respectively, we found that conditions which promote the interaction of Ca^{2+}-ATPase molecules leading to the formation of crystalline arrays

slightly diminish the magnitude and duration of membrane potential generated by ion substitutions (Beeler et al., 1983). The effect of vanadate-induced crystallization on membrane potential implies a slight increase in the permeability of sarcoplasmic reticulum for ions in the crystalline state. It is not known whether this is due to the stabilization of the E_2 conformation of the enzyme or to the development of crystalline arrays. The physiological significance of ATPase-ATPase interactions in the regulation of the permeability of sarcoplasmic reticulum remains to be further explored.

Table 1. Effect of membrane potential generated by K^+ gradient and valinomycin upon the crystallization of Ca^{2+}-ATPase.

Set #	Conditions	+ valinomycin Crystallization index	- valinomycin Crystallization index	Expected Potential (+ val)
1	Na $MeSO_4$(0) → K $MeSO_4$(5)	41.65	4.05	+
2	K $MeSO_4$(0) → Na $MeSO_4$(5)	3.96	1.22	-
3	K $MeSO_4$(0) → K $MeSO_4$(5)	10.04	--	0
4	Na $MeSO_4$(0) → Na $MeSO_4$(5)	4.62	--	0

Sarcoplasmic reticulum vesicles (10 mg protein/ml) equilibrated in media containing 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA and either 0.15 M Na-methanesulfonate (sets 1 and 4) or 0.15 M K-methanesulfonate (sets 2 and 3) were diluted into media containing 5 mM Na_3VO_4 and 0.15 M Na-methanesulfonate (sets 2 and 4) or 0.15 M K-methanesulfonate (sets 1 and 3) with or without 1 μM valinomycin. The concentration of Na_3VO_4 is indicated by (0) and (5), corresponding to zero and 5 mM Na_3VO_4 respectively. Negative staining was initiated 15 seconds after dilution. Crystallization index is the fraction of the vesicles with crystalline regions on their surface expressed as percent of the total number of vesicles (usually 300-800) in random fields of electron micrographs. Significant increase in the rate of crystallization was observed in set 1 in the presence of valinomycin due to the generation of inside positive potential. Nigericine (1 μM) in the dilution medium abolished this effect of valinomycin (from Dux and Martonosi, 1983c).

The effect of proteolysis upon the formation of Ca^{2+}-ATPase crystals.

Tryptic cleavage of the Ca^{2+} transport ATPase of the T_1 site into two major fragments of 57,000 (A) and 52,000 (B) daltons did not interfere with the vanadate-induced formation of membrane crystals, indicating that the two proteolytic fragments interact with preservation of the conformation required for crystallization (Fig. 10) (Dux and Martonosi, 1983d). This is consistent with earlier observations that limited cleavage of the enzyme by trypsin did not interfere with ATPase activity and Ca^{2+} transport (Herrmann and Shamoo, 1982).

The ability of Ca^{2+}-ATPase to crystallize was lost after further cleavage of the A fragment at the T_2 site into the A_1 (34,000 dalton) and A_2 (23,000 dalton) subfragments that is known to be accompanied by loss of Ca^{2+} uptake. Vanadate (0.1 - 5 mM) inhibited the secondary cleavage of Ca^{2+}-ATPase by trypsin at the T_2 site; the susceptibility of the T_2 sites

is influenced either by the conformation of the enzyme or by the formation of crystal lattice.

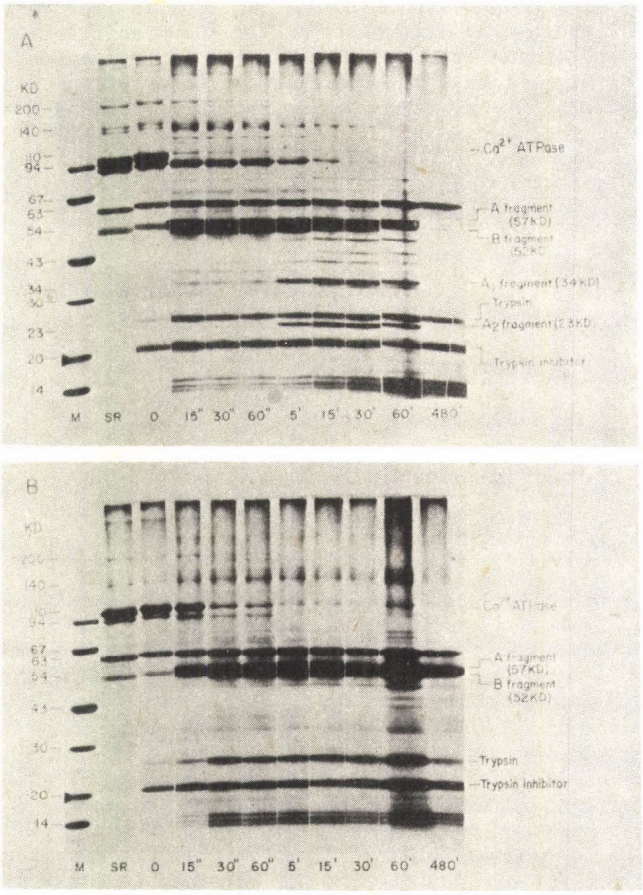


Fig. 10. Tryptic hydrolysis of sarcoplasmic reticulum.
Sarcoplasmic reticulum vesicles (1 mg protein per ml) were digested with trypsin (0.05 mg/ml) in a medium of 0.1 M KCl, 10 mM imidazole, 0.5 mM EGTA, and 5 mM $MgCl_2$, without (Panel A), or with 5 mM Na_3VO_4 (Panel B), at 25°C, for times ranging from 15 seconds to 480 minutes. The digestion was stopped by the addition of soybean trypsin inhibitor (0.1 mg/ml). To samples labeled "0" trypsin and trypsin inhibitor were added together. SR, trypsin-free control sarcoplasmic reticulum. M, molecular weight marker. Panel A: sarcoplasmic reticulum without vanadate treatment. Panel B: prior to trypsin digestion the preparation was exposed to 5 mM Na_3VO_4 in 0.1 M KCl, 10 mM imidazole pH 7.4, 0.5 mM EGTA and 5 mM $MgCl_2$ at 2°C for 36 hours (from Dux and Martonosi, 1983d).

Ca^{2+}-ATPase crystals in sarcoplasmic reticulum of fast and slow skeletal and cardiac muscles. The proportion of vesicles containing Ca^{2+}-ATPase crystals in microsome preparations isolated from rat muscles of different fiber types (semimembranosus, levator ani, extensor digitorum

longus, diaphragm, soleus, heart) correlates well with the Ca^{2+}-ATPase content and Ca^{2+} modulated ATPase activity (Dux and Martonosi, 1983e). This implies that the concentration of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes of fast and slow skeletal or cardiac muscles differs only slightly and the low Ca transport activity of crude microsome preparations isolated from red skeletal and cardiac muscles is due to the presence of large amount of contaminating non-SR membrane elements.

Since the crystalline arrays of Ca^{2+}-ATPase extend over much of the surface of the vesicles, the ATPase content of sarcoplasmic reticulum membranes in adult muscles of diverse fiber-type composition is close to physical saturation. Therefore the sarcoplasmic reticulum content of any given muscle in adult animals is regulated by changing the total amount of sarcoplasmic reticulum of relatively constant ATPase content, rather than by changing the concentration of Ca^{2+}-ATPase in the membrane. As the Ca^{2+} transport ATPase is cotranslationally synthetized on polysomes bound to the surface of the sarcoplasmic reticulum (Martonosi, 1982a) its synthesis and insertion may be inhibited when the membrane is fully saturated by the enzyme. The average distance between ATPase molecules in the membrane is only about 100 Å; therefore it is possible that the hydrophilic head portions of the ATPase molecules interfere with the attachment of the polysomes to the membrane, causing the inhibition of ATPase synthesis when the ATPase concentration rises above a certain level. If this hypothesis is correct the sarcoplasmic reticulum content of a muscle may be largely determined by the availability of relatively phospholipid rich and ATPase poor membrane surfaces with attachment sites for Ca^{2+}-ATPase polysomes. The dimensions of the Ca^{2+}-ATPase crystal lattice are similar in SR membranes of different fiber types; therefore if structural differences exist between "isoenzymes" of Ca^{2+}-ATPase these are not reflected in the crystal-lattice.

Membrane crystals of Ca^{2+}-ATPase in sarcoplasmic reticulum of normal and dystrophic human, mouse and chicken muscle. The dimensions of the Ca^{2+}-ATPase crystals are also similar in sarcoplasmic reticulum membranes isolated from normal and genetically dystrophic human, mouse and chicken muscles (Dux and Martonosi, 1983f). The slightly lower Ca^{2+} transport activity of microsomes isolated from dystrophic muscles is attributable to greater contamination of dystrophic microsome preparations by membranes of non-sarcoplasmic reticulum origin (Martonosi, 1982b).

PERSPECTIVES

The crystallization of Ca^{2+}-ATPase in sarcoplasmic reticulum membranes and reconstituted ATPase vesicles in the presence of vanadate and lanthanide ions opens a powerful new approach for the analysis of the three-dimensional structure of the Ca^{2+}-ATPase.

If the different crystal forms obtained with vanadate and lanthanide ions reflect the different conformational states of the enzyme in successive stages of the transport cycle we may soon be able to obtain information about the structural changes that represent the physical basis of Ca^{2+} translocation. As refinement of the three-dimensional structure to atomic dimensions is not likely to be achieved by electron microscope analysis of two-dimensional crystals, techniques are being developed in our laboratory for the formation of three-dimensional crystals of Ca^{2+}-ATPase, that are suitable for x-ray diffraction studies.

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DISCUSSION

KÖVÉR:

My congratulations on your very excellent lecture. My questions are: Can you substitute vanadate for other inorganic anions (molybdate, wolframate, etc.)? Is the conversation of vanadate to vanadyl of importance in this process? What is the role of dehydration in the crystallization of ATPase? Can crystallization be induced by vanadate using SR prepared from embryonic muscle which has some different functional characteristics?

MARTONOSI:

Vanadate may be substituted by inorganic orthophosphate without major differences in crystal structure. Other inorganic anions were not tried.

Vanadyl formation is not likely to major role in the process.

L. De Meis clearly demonstrated that phosphorylation of the enzyme by inorganic orthophosphate is influenced by the hydration level of the environment. I presume this to be a local effect on the active site. It is not known whether the hydration level changes during the course of our experiment, and whether such changes affect the sites involved in ATPase-ATPase interactions.

The sarcoplasmic reticulum of embryonic skeletal muscle indeed has much lower Ca^{2+} transport activity than the SR of adult animals. This is attributable largely to a lower concentration of Ca^{2+} ATPase in embryonic SR. The ATPase-ATPase interactions required for crystallization are probably dependent on ATPase concentration, since the process appears to be a form of fibrous condensation. In fact there may be a "critical" minimum ATPase concentration that is required for crystallization. This very interesting possibility is currently under investigation by Dr. László Dux at the Institute of Biochemistry, School of Medicine, University of Szeged.

IKEMOTO:

First, I should like to congratulate on your great achievement in the field. I have a question: what the image analysis shows seems to be 2n-mer. What is the evidence that made you decide "dimer"?

MARTONOSI:

The evidence is based entirely on crystallographic arguments. The space group of the lattice is P2; therefore only one of the 17 possible 2 sided plane groups can describe the distribution of matter within the crystal, and this is the dimer.

IKEMOTO:

In the case then, how could we conceive the fact that in the intact system the Ca^{2+}-ATPase oligomer appears to be tetramer.

MARTONOSI:

Association of dimers into tetramers is a likely possibility and under conditions existing physiologically such tetramers may be the favoured species. This would be certainly consistent with the existence of 85 Å intramembranous particles demonstrated by freeze-etch electron-microscopy.

Obviously further work is needed to characterize the monomer - oligomer equilibrium in native sarcoplasmic reticulum.

KEPES:

Since vanadate brings all of the enzyme in the E_2 form the hypothesis of an alternate phase of the two monomers in the functional cycle is out of question. What would be in these conditions the functional role of the dimeric structure and in particular the alternate orientation of the monomers.

ATPase molecules (and therefore free of ATPase crystals). Both explanations are probably applicable, perhaps with different weight under different conditions.

MONTECUCCO:

On the basis of the finding that the Ca^{2+}-ATPase is dimeric in your membrane crystals you suggest a physiological role for the dimer. How relevant is this finding since all membrane proteins (with the exception of bacteriorhodopsin), for which 2-D crystals have been obtained appear as dimers?

MARTONOSI:

I carefully emphasized throughout my talk that the functional significance of ATPase oligomers is entirely unclear. The dimeric unit structure of Ca^{2+}-ATPase derived from the analysis of the crystals refers merely to the structural evidence that I presented, although it is consistent with earlier indications from electronmicroscope, ultracentrifuge, exclusive chromatography, and fluorescence energy transfer data that suggest the presence of ATPase oligomers in native or reconstituted sarcoplasmic reticulum membranes.

It is true, that dimeric unit structures are frequent among membrane proteins. This may reflect some hitherto unrecognized common structural features among them, and certainly does not make a physiological role for ATPase dimers less plausible. Clearly further studies are needed to establish the physiological significance of ATPase oligomers in Ca^{2+} transport.

MARTONOSI:

The proposition that all enzyme molecules are converted by Na_3Vo_4 into the E_2 conformation is consistent with this proposed role of vanadate ion as an analog of inorganic phosphate; this is supported by the inhibitory effect of vanadate on the tryptic digestion of the enzyme at the T2 site, that extends to all enzyme molecules.

In my opinion, existing experimental evidence is not sufficient to conclude that Ca^{2+} transport involves an alternating site mechanism. At any rate the crystalline structure is enzymatically inactive although this inactivation can be reversed by the removal of Na_3Vo_4 and the readdition of Ca^{2+} Mg^{2+} and ATP.

Since vanadate and inorganic phosphate induce similar crystal forms of the Ca^{2+}-ATPase under conditions which resemble those occurring during reversal of Ca^{2+} transport, it seems likely that the dimeric structure induced by vanadate is related to one of the enzyme forms occurring during physiological Ca^{2+} transport.

SZABÓ:

Can you explain the failure of forming regular crystals at the ends of the tubules on the basis of the electron density maps you obtained?

MARTONOSI:

Two explanations can be offered:

1./ The extended crystal lattice is most readily accommodated by cylindrical surface contours due to a special ("wedge-shape") geometry of the Ca^{2+}-ATPase molecules. There is good evidence that on spherical surfaces the crystal lattice is less organized and on concave surfaces it is absent. The hemispheric surface contours located at the end of tubules may interfere with crystallization.

2./ It is not excluded that the crystalline arrays draw together ATPase molecules from a larger area than they occupy, leaving the end region of the tubules free of

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LIPID-PROTEIN INTERACTION IN THE MITOCHONDRIAL ATP-SYNTHASE

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The ATP-synthase complex uses the energy associated with a transmembrane proton gradient to form ATP from ADP and inorganic phosphate. This enzyme can be isolated from bacteria, chloroplast and mitochondria (Sebald 1977, Fillingame 1980, Capaldi 1982). In all these systems it is formed by two separable parts: a) a hydrophilic portion termed F_1, composed of five different subunits indicated in order of decreasing M_r $\alpha,\beta,\gamma,\delta$ and ε; b) a hydrophobic sector termed F_o, which forms a transmembrane proton channel. Bacterial F_o is formed by three different polypeptides, while the chloroplast and mitochondrial systems appear to be more complex. The mitochondrial F_o binds the specific antibiotic inhibitor oligomycin. F_1 possesses an ATPase activity, which is sensitive to oligomycin only when F_1 is bound to F_o. Oligomycin sensitivity is a most sensitive parameter of a correct coupling between F_1 and F_o (Houstek et al. 1982). The activity and oligomycin-sensitivity of the ATP-synthase complex is dependent on and modulated by lipids (Dabbenisala et al. 1974, Bruni et al. 1975, Cunningham and George 1975, Cunningham and Sinthusek 1979). These studies have shown that: a) unsaturated lipids support a higher activity than the saturated counterparts do, b) maximal activation is obtained only in the presence of negative lipids, c) detergents induce loss of oligomycin sensitivity.

Recently Johannsson et al.(1981) have been able to test the effect of another parameter: the lipid bilayer thickness. They have prepared a homologous series of unsaturated lecithins of varying fatty acid chain length from 12 to 23 carbon atoms. These phospholipids are expected to form bilayers of increasing thickness. Fig. 1 shows schematically the structure of such lipids. It is noteworthy that all these lecithins show transition temperatures well below that of the enzymatic assay.

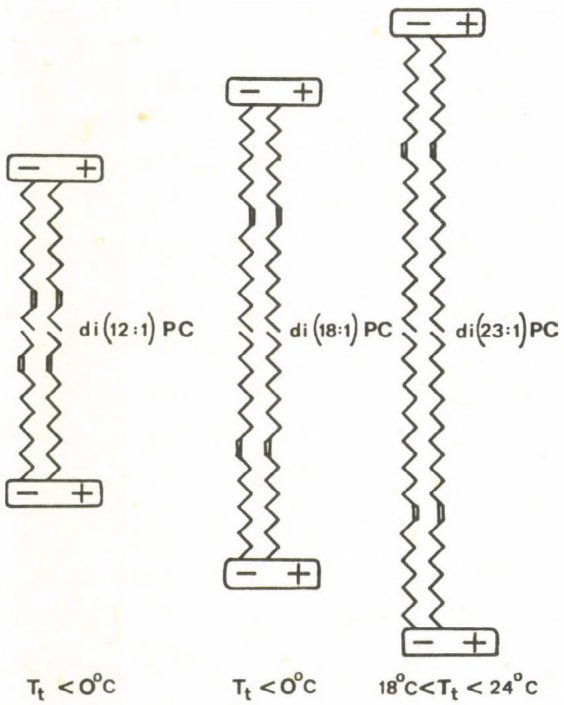

Fig. 1. Shematic structure of three of the lecithins prepared by Johannsson et al. (1981).

With each of these lecithins we have prepared a reconstituted lipid-protein complex with the ATP-synthase prepared either with the method of Galante et al.(1979) or with the method of Serrano et al.(1976). Fig. 2 shows how the oligomycin-sensitive ATP-ase activity varies in function of the fatty acid chain length of the phospholipid used.

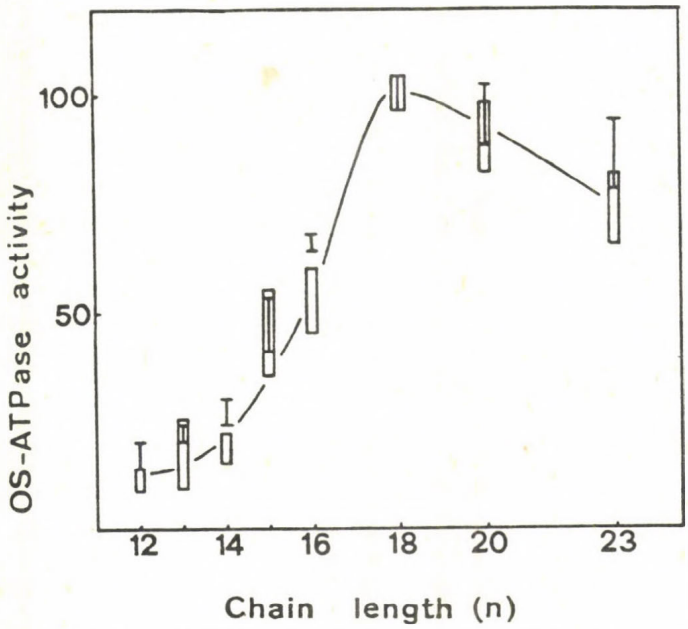

Fig. 2. Effect of lecithin hydrocarbon chain length on the oligomicin-sensitive ATPase activity of beef heart mitochondrial ATP-synthase prepared according to Galante (1979) (I) or to Serrano (1976) (▯). Activities are expressed as percentage of that found with the 18 carbon atom lecithin. Other conditions as in Montecucco et al. (1982).

Clearly with both enzyme preparations maximal activity was found with the 18 carbon atoms lecithin, while thinner or thicker bilayers were incapable to support a high activity. The same result was found with cytochrome c oxidase (Montecucco et al. 1982). These results together with those of Johannsson et al.(1981) support the model shown in Fig. 3. The hydrophobic sector of a transmembrane protein is vertically delimited both at its upper and lower ends by two collars of polar amino acid residues, which interact with the polar head-group of phospholipids. The central part is composed mainly of uncharged residues, which interact with the fatty acid portion of lipids. When the hydrophilic and

Fig. 3. Shematic model of the interaction of the hydrophobic sector of a transmembrane protein with lipid bilayers of different thickness.

and hydrophobic surface portions of the hydrophobic sector do not match their lipid counterpart this will result in a low enzymatic activity. The maximal activities exhibited by both mitochondrial enzymes with the 18 carbon atom lecithin suggest that the polypeptides exposed to lipids can be formed by stretches of 18-20 amino acid residues arranged in an α-helical configuration as proposed for the subunit II of cytochrome c oxidase and subunit b of the F_o of the E. coli ATP-synthase (Bisson et al. 1982, Hoppe et al. 1983).

A more direct and informative approach to study the hydrophobic domain of membrane protein is the hydrophobic photolabelling with photoreactive phospholipids. Fig. 4 shows the structural formulae of the photoactivable lecithins prepared by Bisson and Montecucco (1981). These probes contain the photoreactive nitro-aryl-azido group, which, on illumination with long wave non-protein damaging U.V. radiations, is converted to a nitrene. This reactive intermediate is capable of reacting with a neighbouring molecule, thereby forming a stable covalent adduct. These probes bear the photoreactive group at two different levels of the fatty acid chain in order to probe two different depths of the membrane. They are made highly radioactive because all the three methyl groups of the choline moiety are labeled with ^{3}H or ^{14}C. This allows their use in trace amounts in order to

minimally perturb the biological system under study. Moreover this reduces self quenching.

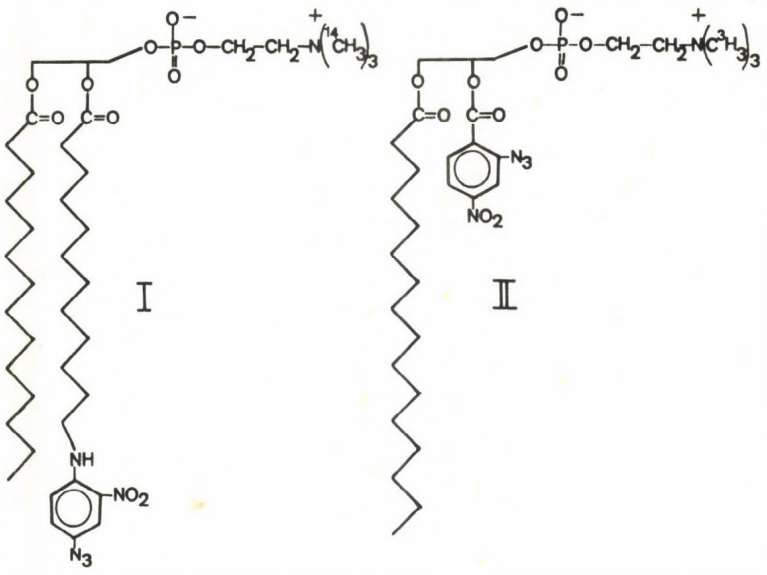

Fig. 4. Structural formulae of the photoreactive lecithins used to probe the structure of the hydrophobic domain of the ATP-synthase.

This method leads to the radioactive labelling of the polypeptides exposed to lipids, which can then be identified by SDS-gel electrophoresis or chromatography (Bisson and Montecucco 1981). Fig. 5 shows the patterns of labelling with the two probes of the beef heart mitochondrial ATP-synthase prepared according to Serrano et al. (1976) and Galante et al. (1979). From the Coomassie Blue staining profiles (panels A and D) it is apparent the complexity of this enzyme, composed of 16 polypeptides, is apparent. In addition to the five F_1 subunits and OSCP (oligomycin-sensitivity conferring protein) there are some ten polypeptides, which are regularly found in different preparations of this complex. New procedures for its isolation will be devised, which will lead to a simpler polypeptide composition, because very probably not all the components are true subunits of the

ATP-synthase. The Serrano et al. preparation contains also a large amount of the adenine nucleotide carrier (29 Kd).

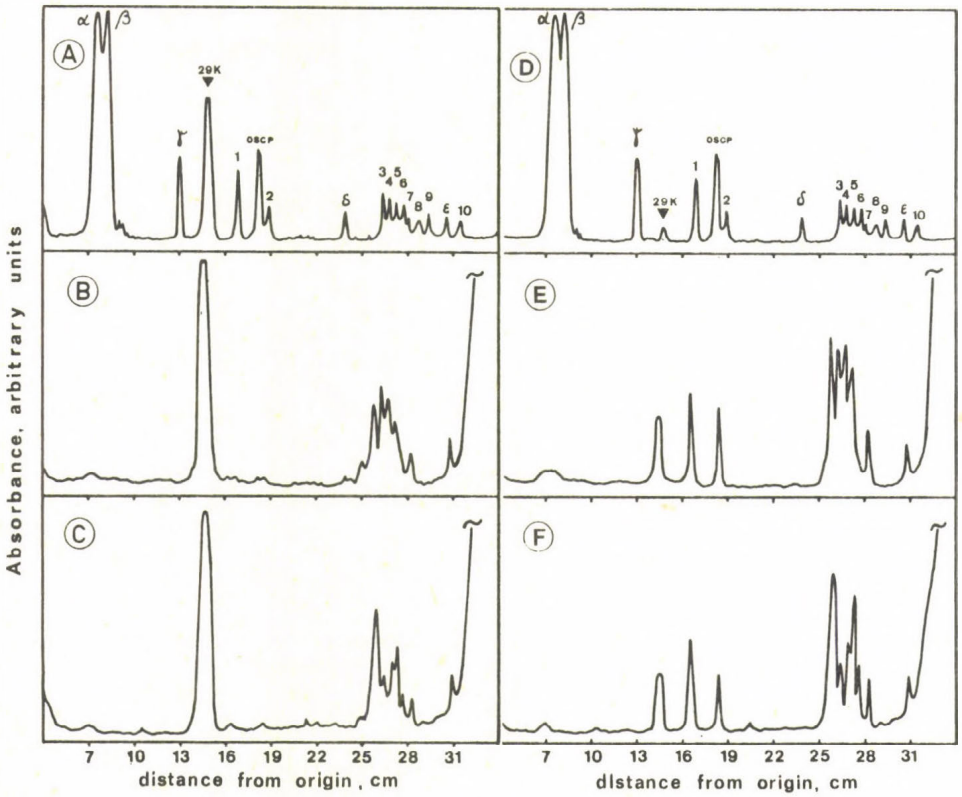

Fig. 5. Coomassie Blue stained profile of a SDS-polyacrylamide gel of the Serrano et al. (panel A) and of the Galante et al. (panel D) preparations of the mitochondrial ATP-synthase run as in Montecucco et al.(1983). Panels B and E report the patterns of labelling, determined by fluorography, with probe I and panels C and F with probe II.

In both preparations none of the five F_1 subunits are labelled. This result supports the idea that F_1 is largely external to the membrane. In the enzyme prepared according to Serrano et al. polypeptides F_o 3, 4, 5 and 6 were labelled by both the deep (I) and the shallow (II) probes together with the 29 Kd band. F_o7 was labelled only with the shallow probe. The preparation of Galante et al. (1979) is largely devoid of the 29 Kd polypeptide. This causes a great change in the profile of labelling. As we

expected much less radioactivity is now associated with the 29 Kd band. Also F_o1 and 2 are labelled, while F_o8 is more labelled than before (see E and F of Fig. 5). It appears that the removal of the adenine nucleotide carrier exposes to lipids F_o1 and 2. This would suggest a proximity relationship in the membrane between the adenine nucleotide carrier and polypeptides F_o1, 2 and perhaps 8. It is noteworthy that a close correlation exists between F_o1 and 2 and subunits a and b of the F_o sector of the E. coli ATP-synthase suggesting that they are true subunits of the mitochondrial ATP-synthase (Walker et al. 1983).

In conclusion the studies briefly described here suggest that the hydrophobic domain of the mitochondrial ATP-synthase: 1) has a fixed vertical dimension determined at both its upper and lower ends by two hydrophilic regions interacting with the head groups of phospholipids, while the intermediate protein surface is hydrophobic and interacts with the fatty acid portion of lipids; 2) it appears to be formed by several different polypeptides; 3) F_o1 and 2, which are part of the lipid-protein boundary of the Galante et al. complex, appear to interact directly with the adenine nucleotide carrier. This opens the possibility that this proximity relationship may have a physiological significance.

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DISCUSSION

SANADI:

I am very pleased to see your results with the photo active deep and surface probes, since we have obtained similar results with a series of carboxyl maleimides in which the carboxyl and maleimide groups were separated by 1 to 12 methylenes. The H^+-ATPase, assayed by P_i-ATP exchange and oxonol binding reactions, was the most sensitive when the polar and non-polar groups were separated by 10 methylenes. With these reagents, the carboxyl stays on the bilayer surface and the maleimide penetrates into the hydrophobic region. The correspondence of your observation that the b subunit of E.coli F_0 has an -SH in the hydrophobic region with our findings that there is an hydrophobic-SH in coupling factor B, which is buried in the membrane lends further support to our published speculation that F_B may be related to the b subunit of E.coli F_0.

AZZI:

Do you have evidence that the lipid-protein complexes made with phospholipids of different length contain bi-layer structures?

MONTECUCCO:

The lipid-protein complexes were formed with the cholate-dilution method. Excess lipid-cholate mixtures were mixed with the protein at $0^{\circ}C$ and after few minutes of incubation were diluted in two steps two thousand times. This way leaky liposomes containing the protein are formed. The liposomes have a bilayer structure.

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MECHANISM OF SIGNAL TRANSMISSION IN ACTIVATED LYMPHOCYTES

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Resting lymphocytes are activated by antigens or mitogens to differentiate, and simultaneously to grow and divide. Although to proceed to cell division e.g., an individual lymphocyte clearly requires additional stimuli such as interleukin 2, it appears well established that the activation process is initiated by the binding of an activating ligand to receptors of the plasma membrane. How, in molecular terms, the ligand receptor interaction triggers a cellular response - still remains unsolved. Shortly after the binding of a mitogen - stimulation by mitogens has provided the predominant model for studying molecular events in lymphocyte activation - the plasma membrane undergoes numerous changes including receptor reorganization, physicochemical changes, alterations in the permeation of various substances, notably also ions, or changes in the activity of membrane bound enzymes. Many of these changes in the activation occur early enough to be associated with a purported triggering event (see Resch 1976, Resch 1979, Wedner and Parker 1976, Hume and Weidemann 1980, Johnstone and Crumpton 1982).

A possible explanation for this pleiotypic plasma membrane response is the assumption that a receptor linked amplification is operative in the plasma membrane underlying the many functional changes observed. On the basis of preliminary evidence we have suggested earlier that the metabolism of membrane phospholipids may represent its molecular counterpart (Resch and Ferber 1976, Ferber and Resch 1977). This could be corroborated

by more recent findings. Thus this contribution is addressed specifically to the question how molecular events possibly responsible for signal transmission in activated lymphocytes are coupled to activating receptors by plasma membrane phospholipids. Due to our limited experience, we will deal exclusively with the activation of T lymphocytes by mitogens.

Membrane phospholipids change rapidly in stimulated lymphocytes

Also in resting lymphocytes membrane phospholipids are in a dynamic equilibrium, as evidenced by a continuous incorporation of labelled precursors in the absence of net synthesis. The separate turnover of phospholipid fatty acids - due to a deacylation-reacylation reaction (Hill and Lands 1970) - by far exceeds the synthesis de novo of different phospholipids measured by the incorporation of long chain fatty acids (Ferber and Resch 1977). The physiological role of this pathway can only be appreciated if one considers the possibility that cleaved and reincorporated fatty acids are not identical. Thus it provides a powerful mean to change the phospholipid fatty acid composition of cellular membranes. As many membrane functions depend on the nature of the phospholipid matrix, and especially also on its fatty acid composition, the deacylation-reacylation reaction appears to be a good candidate providing the putative amplification device operating within the plasma membrane of activated lymphocytes.

When resting lymphocytes were stimulated with different mitogens such as concanavalin A (conA) the incorporation of an unsaturated fatty acid such as oleate (C18:1) into phospholipids increased (Resch and Ferber 1972), much more prominently when the incorporation was followed in a cell fraction enriched in plasma membranes (Resch et al. 1972). In recent experiments, where the incorporation of all major fatty acids present in lymphocytes into individual phospholipids of conA stimulated rabbit lymphocytes was measured, it became apparent that incorporation was stimulated asymmetrically for different fatty acids (Rode et al. 1982; Brennecke and Resch, manuscript in preparation).

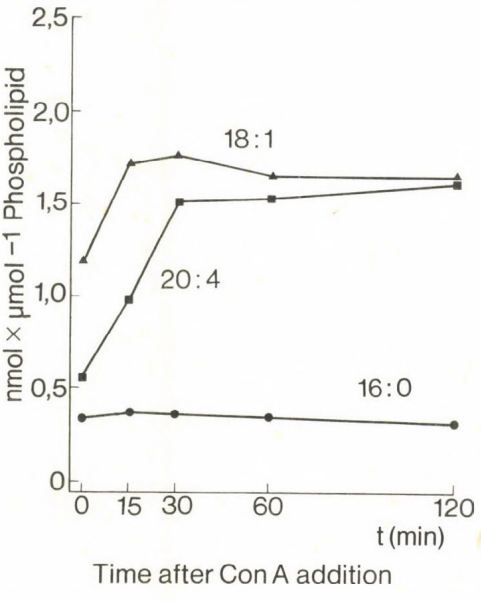

Fig. 1:

Initial incorporation rates into total phospholipid of plasma membranes from rabbit thymocytes

Thymocytes ($5x10^7$ cells/ml) in RPMI 1640 were stimulated with concanavalin A (10 µg/ml) and pulsed with ^{3}H-arachidonate (20:4), ^{3}H-palmitate (16:0) or ^{14}C-oleate (18:1) either (10 nmol/ml) for the last 5 min of stimulation time indicated.

As it can seen in Fig. 1, the initial incorporation rates for arachidonate (C20:4), and to a more less extent for oleate (C18:1) increased, reaching a new steady state after 30 min, whereas those for palmitate (C16:0) remained unchanged. The incorporation of arachidonate into all major membrane phospholipid species increased, within the first 30 min of conA stimulation most prominently into phosphatidylcholine and phosphatidylinositol. Whereas the increased incorporation of arachidonate into the latter phospholipid was similar in all cellular membranes, the incorporation into the main phospholipid phosphatidylcholine was increased exclusively in the plasma membrane. This suggested that changes occuring early after mitogen activation in plasma membrane phosphatidylcholine are an immediate consequence of the ligand binding.

The mitogen induced changes of the turnover of the phosphatidylcholine fatty acids were directed, leading to a preferential incorporation of polyunsaturated fatty acids. As a

consequence, the content of these fatty acids in membrane phospholipids was found to be increased in total cell extracts of stimulated lymphocytes (Ferber et al. 1975). As highly sensitive methods such as glass capillary gas chromatography became available, recently we could measure the fatty acid composition of individual phospholipid species in plasma membranes isolated from conA stimulated rabbit thymus lymphocytes (Table 1).

Table 1

CHANGES IN THE FATTY ACID COMPOSITION OF PLASMA MEMBRANES FROM ConA-STIMULATED RABBIT THYMUS LYMHPOCYTES a)

	Relative Distribution										
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20_{us} b)	20:4	22_{us} c)
PHOSPHATIDYL-CHOLINE (PC)											
control	0.6	54.9	1.3	12.4	16.4	10.9	0.3	0.1	1.8	3.1	0.5
conA	0.7	38.0	1.0	12.4	19.7	17.6	0.3	0.2	2.6	7.6	1.3
PHOSPHATIDYL-ETHANOLAMINE (PE)											
control	-	12.6	4.2	35.4	7.7	1.5	1.9	0.7	2.5	22.1	10.9
conA	-	4.0	3.4	26.2	8.5	4.2	0.5	0.2	2.4	34.2	16.9
PHOSPHATIDYL-INOSITOL/SERINE (PI/PS)											
control	-	3.5	0.6	71.4	8.0	1.9	1.0	0.4	0.8	10.1	2.0
conA	-	2.8	0.5	59.0	8.8	3.2	0.2	0.2	1.7	21.3	2.6

a) $5x10^7$ calf thymocytes/ml Hepes buffered RPMI were stimulated for 4 hours with 5 µg/ml conA, then plasma membranes were isolated and analyzed

b) unsaturated, 1 to 3 double bounds

c) polyunsaturated, 2 to 5 double bounds

After 4 hours' stimulation in the absence of exogenous fatty acids in phosphatidylcholine the content of linoleate (C18:2) and arachidonate (C20:4) approximately doubled on the expense of the major saturated fatty acid palmitate (C16:0). Similar changes were found in a mixture of phosphatidylinositol and phosphatidylserine, with the exception that the content of stearate (C18:0), the major saturated fatty acid here, decreased.

In phosphatidylethanolamine in addition to linoleate and arachidonate the content of polyunsaturated C22 fatty acids was also considerably enhanced (Goppelt and Resch, manucript in preparation).

As a physicochemical counterpart of these changes the membrane fluidity increased in membranes from conA stimulated cells, also in the lipid phase extracted from them (Ferber et al. 1974). Recently using fluorescence polarization of diphenylhexatriene the decrease in fluidity of plasma membranes was apparent after 1 hour of stimulation (Goppelt, Urbach and Resch, manuscript in preparation).

Although the detailed enzymatic mechanism is not fully understood, the fatty acid changes in plasma membranes from stimulated lymphocytes appear to be implicated in the properties and mode of activation of acylcoA lysolecithin acyltransferase, the enzyme which is responsible for transferring (activated) fatty acids into lysophospholipids. This enzyme is present with high specific activities in the plasma membrane of lymphocytes (Ferber et al. 1972, Kaever et al., submitted to J. Biol. Chem.).

Upon stimulation with conA, the specific activity towards all fatty acid-coA's was increased, however, with a greater stimulation towards polyunsaturated fatty acids (Ferber et al. 1976, Szamel and Resch 1981). While the K_m values for unsaturated fatty acids were not changed, the K_m for palmitoyl-coA drastically increased in membranes from activated cells. To opearate with cellular membranes, lysolecithin acyltransferase requires the continuous generation of lysophosphatidylcholine as acceptor for activated fatty acids. In many cells - e.g. mononuclear phagocytes - it is formed by phospholipase A. Although phospholipase A activity was described in spleen cells (Hirata et al. 1980), attempts to detect this enzyme in macrophage deprived lymphocytes such as thymus lymphocytes have so far failed (Trotter et al. 1982; Goppelt, unpublished). Recently Trotter and Ferber (1981) suggested that the back reaction of lysolecithin acyltransferase can also constitute the phospholipid deacylation reaction, as it has also been suggested

for other cells (Irvine and Dawson 1979). The markedly increased K_m for palmitoyl-coA in membranes from stimulated lymphocytes is compatible with an increased saturated fatty acid specific deacylation by lysolecithin acaltransferase. Thus it is intriguing to speculate that the regulation of one enzyme, lysolecithin acyltransferase, is responsible for the specific plasma membrane fatty acid changes found in activated lymphocytes.

AcylcoA:lysolecithin acyltransferase is part of a receptor-associated plasma membrane domain

The proposed role of phospholipids as a signal amplification device requires to define the coupling of mitogenic receptors to enzymes of the phospholipid turnover within the plasma membrane, specifically to lysolecithin acyltransferase. In the case of mitogen stimulation of T lymphocytes discussed here, this is hampered at present, as the molecular nature of the mitogen receptor(s) has not been established definitely, due to the fact that the most commonly used mitogens, i.e. lectins, bind to a large number of glycoproteins and glycolipids. Some characteristics, however, have emerged, which distinguish the triggering receptors from biologically inert mere binding sites. Different T cell mitogens such as various lectins, oxidizing agents or antibodies share the same glycoprotein receptors (Resch 1976). The most important property of triggering mitogen receptors distinct from bulk binding sites consists in their higher affinity for a mitogen (Allan and Crumpton 1973). Making use of this property we have isolated fragments of the plasma membrane which, within the framework of the premise, bear the triggering receptors. Calf thymus lymphocytes as an unlimited source of pure T lymphocytes - similar results were also obtained with rabbit thymus lymphocytes - were disrupted by nitrogen cavitation (Ferber et al. 1972), which releases small plasma membrane fragments immediately forming vesicles with a mean diameter of about 70 nm (Brunner et al. 1978). Highly purified plasma membranes were isolated by means of differential centrifugation and sucrose gradient ultracentrifugation

Fig. 2a

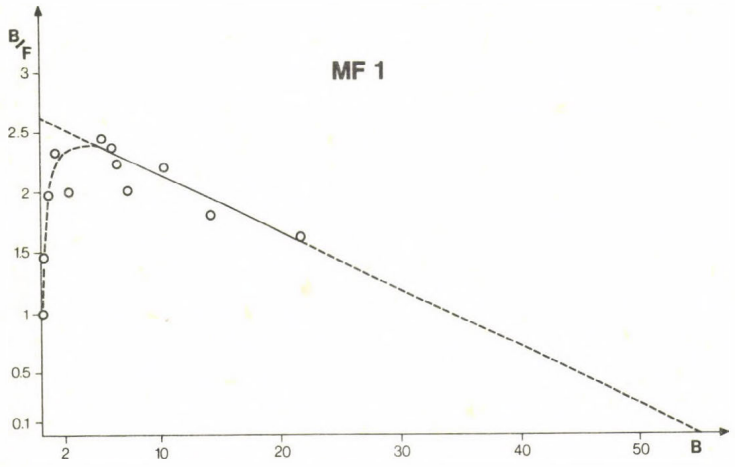

Fig. 2b

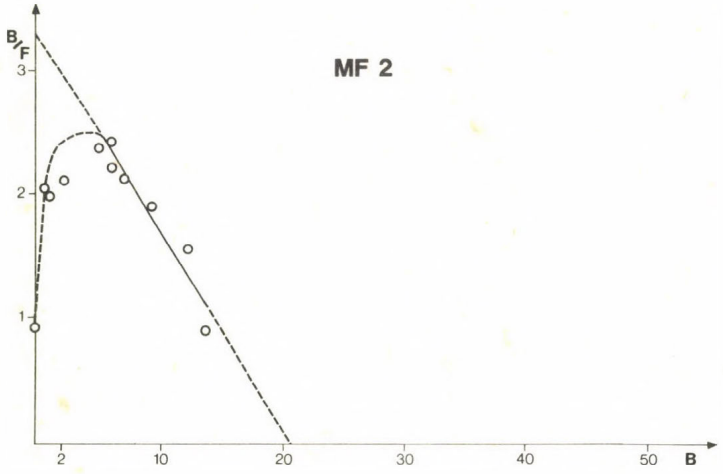

Fig. 2a/b Binding of Concanavalin A to Plasma Membrane Subfractions.

Binding of ^{125}I-labelled concanavalin A to 40 µg/ml membrane protein was measured as described in Resch et al. 1983. The data are plotted according to Scatchard. In their linear parts as indicated by solid lines, the lines were fitted by least square regression.
B = bound concanavalin A (µg/100 µg membrane protein)
F = free concanavalin A (µg/ml)

(Resch et al. 1981). Using affinity chromatography on conA-sepharose, the plasma membranes were separated into two distinct fractions, one eluting freely from the affinity column (MF_1), the second being retained (MF_2) (Brunner et al. 1977, Resch et al. 1981). Extensive control experiments showed that both fractions were derived from the same cell exclusively, originated from plasma membranes and had preserved their original orientation, i.e. proved to be right side-out, thus representing different domains of the plasma membrane of T lymphocytes (Brunner et al. 1977, Resch et al. 1978, Resch et al. 1981, Resch et al. 1983). The fraction retained on conA-sepharose, consisting of about 20 per cent of the total plasma membrane protein, contained less binding sites for conA, which, however, exhibited a several-fold higher (mean) affinity for this mitogen (Resch et al. 1983) (Fig. 2). This strongly suggests that the plasma membrane domains segregating with this fraction, i.e. MF 2, bear the triggering receptors.

Table 2 Distribution of Lysolecithin Acyltransferase in Plasma Membrane Subfractions.

Plasma membranes were separated into MF 1 and MF 2.
MF 1 then was rechromatographed on concanavalin A-sepharose into MF 1' and MF 2'.

	nmol x mg protein^{-1} x min^{-1}, 37°C				
	Plasma membrane	MF 1	MF 1'	MF 2	MF 2'
Oleoyl-coenzymeA:lyso lecithin acyltransferase	3.9	3.5	1.7	7.3	7.3
Arachidonoyl-coenzymeA:lyso-lecithin acyltransferase	15.3	14.3	n.d.	40.0	n.d.
	13.6	n.d.	6.3	n.d.	29.9

The properties of the plasma membrane subfractions are given in the accompanying contribution by Szamel et al. For the present discussion it is important that acylcoA:lysolecithin acyltransferase segregated together with the high affinity receptor suggesting receptor associated domains being present in

the plasma membrane containing this key enzyme of the phospholipid fatty acid metabolism (Table 2).

Substitution of plasma membrane phospholipids with polyunsaturated fatty acids mimicks membrane changes induced by mitogen stimulation

Lysolecithin acyltransferase is present with high specific activities in highly purified lymphocyte plasma membranes (Ferber et al. 1972; Resch et al. 1981; Kaever et al., submitted to J. Biol. Chem.). By the use of this enzyme the composition of plasma membrane phosphatidylcholine could be modified experimentally in a very controlled way. From the precursors lysophosphatidylcholine (=lysolecithin) and coenzymeA-derivatives of different fatty acids phosphatidylcholine was formed, which proved to be exclusively plasma membrane-associated. Control experiments with radioactively labelled precursors documented that experimental conditions could be found allowing a nearly quantitative conversion of the added acylcoA as well as lysophosphatidylcholine. The increase of the content of the saturated fatty acid palmitate (C16:0) in phosphatidylcholine had no measurable effect on enyzme activities. The incorporation of the monounsaturated fatty acid oleate (C18:1) had a small but consistent effect, similar in direction as obtained with the substitution by polyunsaturated fatty acids. The enhancement of the content either of the polyunsaturated fatty acids linoleate (C18:2) or arachidonate (C20:4) markedly modulated plasma membrane-associated enzymes, with a peak effect at a fatty acid substitution increasing the content of polyunsaturated fatty acids by about 12 per cent. As it is shown in Table 1, fatty acid changes of this magnitude occurred in plasma membranes of mitogen activated lymphocytes. At higher incorporation rates enzyme activities reverted to background levels. Upon increasing the content of polyunsaturated fatty acids, the specific activities of $(Na^{+}+K^{+})$ATPase and lysolecithin acyltransferase increased, whereas the activities of Mg^{++} ATPase, γ-glutamyltranspeptidase, or alkaline phosphatase decreased (Table 3) (Szamel and Resch, 1981).

Table 3 Specific Activities of Membrane Bound Enzymes in Fatty Acid Modified Calf Thymocyte Plasma Membranes [1]

plasma membranes modified with	nmol x mg protein^{-1} x min^{-1}, 37°C				
	$[Na^+ + K^+]$ ATPase	Mg^{++} ATPase	Ol.-coA:[2] LL ATase	Ara.-coA:[3] LL ATase	γ-GTase[4]
control	14.5	74.6	4.7	12.4	18.8
palmitate (16:0)	14.9	73.1	4.9	n.d.	18.8
oleate (18:1)	16.4	70.8	5.1	n.d.	16.5
linoleate (18:2)	26.9	47.2	11.1	37.8	7.1
arachidonate (20:4)	26.0	52.2	10.9	41.6	6.6

[1] at a fatty acid substitution resulting in maximal enzyme modulation

[2] oleoylcoA:lysolecithin acyltransferase

[3] arachidonoylcoA:lysolecithin acyltransferase

[4] γ-glutamyl transpeptidase

Plasma membranes were modified enzymatically with different fatty acids and the enzyme activities were determined (Szamel and Resch 1981).

These changes in enzyme activities strikingly mimicked the ones apparent in plasma membranes from mitogen stimulated lymphocytes (Table 4).

Table 4 Specific Activities of Membrane Bound Enzymes in the Plasma Membrane of ConA-Stimulated Thymocytes.

	nmol x mg protein^{-1} x min^{-1}, 37°C	
	Control	ConA [a]
$[Na^+ + K^+]$ATPase	15.20	32.60
OleoylcoA:lysolecithin acyltransferase	3.85	8.02
ArachidonoylcoA:lysolecithin acyltransferase	15.93	39.72
Mg^{++}ATPase	118.10	101.50
Alkaline phosphatase	192.00	104.00
γ-glutamyl transpeptidase	16.80	10.90

a) 10^{10} cells in 200 ml of Dulbecco's modified Eagle's medium were stimulated for 60 min with 10 µg/ml of conA

Thus real changes occurring at membrane sites apart from the receptor in mitogen activated lymphocytes may well be caused by fatty acid changes of the phospholipid membrane matrix.

The role of phospholipids in signal transmission initiating lymphocyte activation (Fig. 3)

On the basis of the evidence presented we propose a mechanism for the initiation of the activation of T lymphocytes, implicating plasma membrane phospholipids in signal transmission. In resting lymphocytes the plasma membrane contains domains formed by a tight association of the triggering mitogen receptors with (at least) two enzymes $(Na^+ + K^+)$ATPase and lysolecithin acyltransferase. The binding of a mitogen to its receptors represents the initial event in the activation process. As an immediate consequence of the ligand-receptor interaction, the receptor linked enzymes are activated.

Due to the activation of lysolecithin acyltransferase, the saturated fatty acids of plasma membrane phospholipids, initially predominantly of phosphatidylcholine, are replaced by polyunsaturated ones. As the newly formed phospholipid molecules with higher degree of polyunsaturation are rapidly dispersed (Rode et al. 1979) the entire membrane matrix is altered, as also evidenced by an increase in fluidity. The increase in the content of polyunsaturated fatty acids in membrane phospholipids modulates other membrane functions such as the activity of plasma membrane bound enzymes. One of such effectors - or several in a concerted action - then creates (or contributes to) the intracellular messenger(s), responsible for cell activation. By linking different membrane functions to the trigerring receptor and thus amplifying a signal within the plasma membrane, we propose that plasma membrane phospholipids form an integral part in the stimulus-response coupling in the activation of lymphocytes.

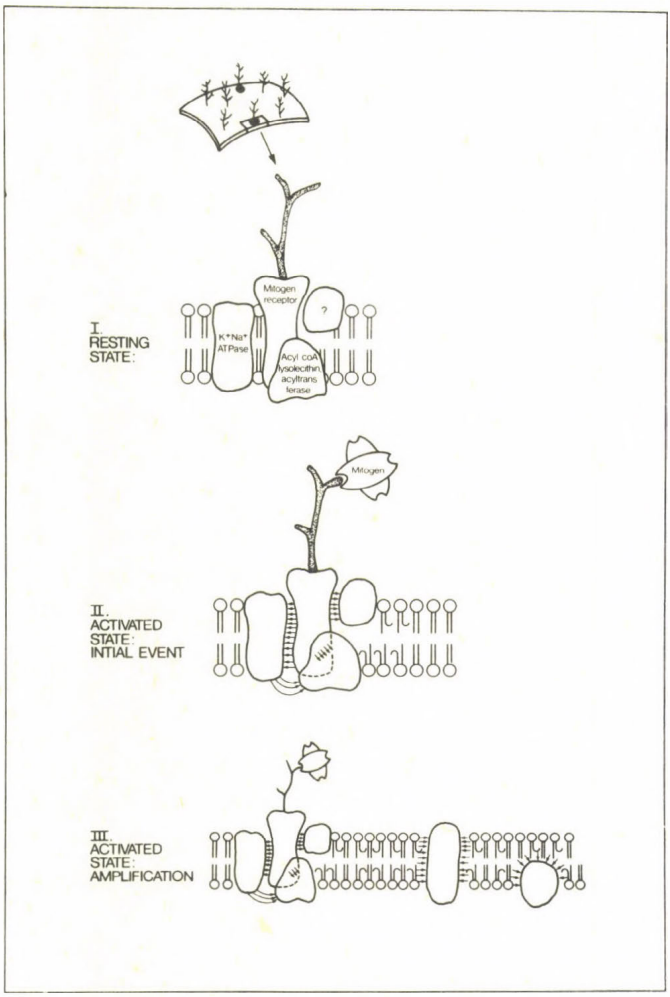

Fig. 3 Mechanism of Signal Transmission in Activated Lymphocytes.

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DISCUSSION

WOJTCZAK:

I wonder whether you may find it worthy to perform an experiment in which either intact lymphocytes or isolated plasma membranes are incubated with liposomes containing a high proportion of "polyunsaturated" phospholipids in the presence of phospholipid-exchange proteins. In such a way you could change the phospholipid composition of the plasma membrane without the involvement of the phospholipid synthesizing system.

RESCH:

Principally it could be possible to change the plasma membrane phospholipid composition by using phospholipid exchange proteins as you suggested. We have also thought of this possibility, but have not performed experiments so far. The use of phospholipid exchange proteins may be especially useful in intact lymphocytes: As our enzymatic method requires initially high concentrations of lysophospholipids in its present form it cannot be applied to intact cells because of the lytic properties of these detergent-like substrates. A possible alternative to the use of phospholipid exchange proteins would be the continuous generation of lysophospholipids by exogenous phospholypase A, however, such a method has not been established.

FONYÓ:

How is the message transmitted from the Con-A receptor to the lysolecithin acyltransferase?

RESCH:

This is one of the crucial questions we can only answer hypothetically. One possibility can be fairly excluded, namely that lysolecithin acyltransferase itself is the receptor, as in cells where it has been isolated (e.g. erythrocytes) the enzyme is a non-glycosylated protein

exposed only to the interior face of the plasma membrane. The most simple mechanism is suggested by the kinetics of its activation (Ferber et al., 1975). Activation of lysolecithin acyltransferase by con-A does not follow simple kinetics, but fits a Hill equation with n=1.8. This can be interpreted that the activation is due to an allosteric transition in which two lectin binding sites cooperate for one activated enzyme entity. In the light of the known fact that mitogenic activation of lymphocytes requires the binding of (at least) divalent ligands, the minimal hypothesis would be that the clustering of the mitogen receptor activates acyltransferase simply by bringing two enzyme sites together. Indeed, as it is discussed in this contribution, receptor and acyltransferase are tightly linked in the lymphocyte plasma membrane supporting strongly this minimal hypothesis.

BHARGAVA:

Your observations are no doubt, interesting. However, what is important is to know where they stand in the hierarchy of events that occur when a resting cell is triggered into the division cycle. In this hierarchy, as I pointed out this morning, we must distinguish between the switch (an early, probably in most cases the first event) that would control this program of the cell cycle, and the programme itself. You seem to believe that the insertion of unsaturated fatty acids in this lipid bilayer through activation of the transferase, is the switch, so what the mitogen needs to do is just this. In that event, if one was able to make the lipid bilayer appropriately rich in poliunsaturated fatty acids in some other way, the cell would be triggered into the cell cycle. One can, in fact, try this today, by fusing (through PEG or Sendai virus) liposomes made rich in polyunsaturated fatty acids, with a resting cell. This fusion, then, should trigger the cell without a mitogen. I wonder if you have considered something of this sort.

RESCH:

As I have pointed out the role we would like to ascribe to plasma membrane phospholipids is to couple putative membrane associated effectors, which finally are responsible for providing an intracellular signal (or second-messenger) to the triggering receptors. This idea closely resembles the mechanism of the second messenger generation in the stimulation of cells by some hormones, the best known example being the regulation of the synthesis of cAMP by β-adrenergic hormones. The major difference is that in contrast to a coupling protein - which links the β - receptor to the catalytic unit of adenylate cyclase - we propose an amplifying metabolic process which changes the matrix of the plasma membrane. The major advantage of this mechanism is that more than one - in principle an unlimited number - of effectors can thus be linked to the receptor, which may be essential for such complicated events as cell differentiation or growth control. In your terminology the mechanism we have proposed would thus be associated intimately with the switch.

With respect to the second part of your question: It is of course a very attractive idea, which we have considered extensively, that the increase in the content of polyunsaturated fatty acids in plasma membrane phospholipids provides the minimal requirement that cells like lymphocytes proceed from a resting to a differentiated state or to division. However, as it has become known within the last few years, the progression of a resting lymphocyte to cell division requires several sequentially acting stimuli. Thus, as it was pointed out, the mechanism we propose, strictly concerns the initiation of this sequence of events leading to mitosis. We are at present setting up the correct experimental models enabling us to study whether the initial triggering event can be provided by simply increasing the content of polyunsaturated fatty acids by means similar to those you suggested.

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FUNCTIONAL INTERRELATIONSHIP BETWEEN $(Na^+ + K^+)$ ATPase AND LYSOLECITHIN ACYLTRANSFERASE IN THE PLASMA MEMBRANE DOMAINS OF LYMPHOCYTES

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After the binding of antigens or mitogens to the receptors of the plasma membrane of lymphocytes a cascade of metabolic changes is initiated leading to growth, differentiation and proliferation of the cells. Mitogen lectins such as concanavalin A (conA) bind to a variety of cell surface glycoproteins and glycolipids (Alexander et al., 1978, Crumpton and Walsh, 1978, Sela, 1980); however, activation is mediated only by a small proportion of receptors, distinct from the bulk binding sites by having a higher affinity for the mitogen (Allen and Crumpton, 1973).

As the triggering process is initiated by the binding of the mitogen to cell surface receptor the critical event leading to cell growth and differentiation must be restricted to the plasma membrane, and by definition it has to occur early. Most efforts so far have been devoted to establishing early membrane changes in the hope of finding the earliest one, which then would be responsible for the metabolic events in activated cells (cf. Resch, 1976, Hume and Weidemann, 1980, Johnstone and Crumpton, 1982). However, by using this approach no clearcut answers have emerged. Thus to get more direct information about the initiation of lymphocyte activation a different approach was used, e.g. the study of the functional topology of the lymphocyte plasma membrane. The aim was to study the micro-environment of the biologically active mitogen receptors.

The model used in these studies consisted of highly purified plasma membranes of thymocytes isolated as described by Resch et al., 1981, Resch et al., 1983 (see also accompanying contribution by Resch et al.) The obtained membrane vesicles were further fractionated by the means of affinity-chromatography on conA-sepharose; two subfractions were obtained, one eluted freely from the affinity column (MF_1), the second was retained and could be eluted by α-methyl-mannoside (MF_2).

A series of control experiments was performed to establish the source of the observed heterogeneity of lymphocyte plasma membrane vesicles. All of the obtained experimental evidence argued

strongly against irrelevant possibilities and is presented in detail elsewhere (Resch et al., 1978, Resch et al., 1981, Resch et al., 1983).

A number of criteria evidenced that both subfractions were derived from plasma membranes of thymocytes:

1. The plasma membranes were highly purified (Resch et al., 1981), and were devoid of β-glucuronidase, acid phosphatase or succinate dehydrogenase, which rules out contamination by intracellular membranes,

2. plasma membranes as well as both subfractions had an identically high cholesterol to phospholipid ratio as a characteristic for plasma membranes (Table 1).

Table 1

Cholesterol and phospholipid content of plasma membrane subfractions

	µmol x mg protein^{-1}		molar ratio- $\frac{\text{Chol}}{\text{Plipid}}$
	Chol[a)]	Plipid[b)]	
Plasma membrane	0.641	1.090	0.588
MF_1	0.638	1.085	0.588
MF_2	0.570	1.077	0.530

a) cholesterol

b) phospholipid

3. Enzymatic surface radioiodination or labelling of surface carbohydrates by tritiated $NaBH_4$ reduction was identical in the subfractions (Table 2).

Table 2

Distribution of surface label in plasma membrane subfractions

	cpm x mg protein^{-1}	
	^{125}I a)	^{3}H b)
Plasma membrane	237 578	283 665
MF_1	240 051	294 827
MF_2	244 564	308 970

a) Intact lymphocytes were surface labelled by lactoperoxydase catalized radioiodination as described by Resch et al. (1983). Then plasma membranes were isolated and fractionated into MF_1 and MF_2.

b) Isolated plasma membranes were treated with neuraminidase and galactose oxydase, the oxydized carbohydrates were reduced with NaB^3H_3 as described by Resch et al. (1983). The labelled plasma membranes were then fractionated into MF_1 and MF_2.

4. In both subfractions a number of plasma membrane bound enzymes showed identical specific activities, including γ-glutamyl-transpeptidase, alkaline phosphatase and Mg^{2+}-ATPase (Table 3).

Although both membrane subfractions share some common properties indicating their exclusive plasma membrane nature differences can also be found. Some membrane specific enzymes showed a clearcut heterogeneous distribution. Thus, (Ca^{2+}+Mg^{2+}) ATPase, (Na^++K^+)ATPase were enriched several fold in MF_2 compared to MF_1. Lysolecithin acyltransferase, the enzyme responsible for the enhanced phospholipid turnover of stimulated lymphocytes, was also present in MF_2 with higher activities compared to MF_1 (Table 3).

Table 3

Distribution of enzyme activities in plasma membrane subfractions

	PM[a]	MF_1	MF_2
	nmol x mg protein^{-1} x min^{-1}, 37° C		
γ-glutamyl-transpeptidase	28,0	30,0	28,0
Alkaline phosphatase	127,0	90,0	135,0
Mg^{2+}-ATPase	60,0	76,0	63,0
$(Ca^{2+}+Mg^{2+})$ATPase	10,5	2,7	15,1
$(Na^{+}+K^{+})$ATPase	18,3	8,2	30,7
K^{+}-dependent p-nitrophenylphosphatase	3,5	0	7,8
OlCoA-LAT[b]	5,0	3,5	8,3
AraCoA-LAT[c]	15,0	9,9	35,1

[a] plasma membrane

[b] OleoylcoA: lysolecithin acyltransferase

[c] ArachidonoylcoA: lysolecithin acyltransferase

These differences were even expanded after rechromatography, allowing in the case of $(Na^{+}+K^{+})$ATPase the contention that this enzyme is exclusively present in the adherent fraction.

The critical difference between the two plasma membrane subfractions consisted in their different affinity for conA: MF_2 exhibited an association constant which was several times higher than that of MF_1 (for details see accompanying contribution by Resch et al.), showing that MF_2 bears the high affinity receptors for mitogens.

In agreement with these results polyacrylamide gel electrophoresis revealed different polypeptide patterns of the two subfractions. Several polypeptides were markedly enriched in the adherent fraction MF_2, the most striking differences were observed in the amounts of 170, 150, 95, 75, 40 and 30 kilodalton proteins. In contrast, several other proteins (45-55 kilodaltons) were preferentially recovered in MF_1. In addition, actin and myosin were detected in equal amounts in both subfractions further substantiating the exclusive plasma membrane origin of the subfractions (Szamel and Resch, unpublished).

Table 4

Specific activities of membrane bound enzymes in plasma membrane subfractions of conA-stimulated thymocytes

	nmol x mg protein^{-1} x min^{-1}, 37° C					
	plasma membrane		MF_1		MF_2	
	control	conA	control	conA	control	conA
(Na^++K^+)ATPase	15.2	32.6	4.9	5.9	23.3	42.6
OleoylcoA: lysolecithin acyltransferase	3.9	8.0	3.2	3.6	7.3	15.5
ArachidonoylcoA: lysolecithin acyltransferase	15.9	39.7	11.9	14.3	31.9	102.0
MG^{++}-ATPase	118.1	101.5	139.1	133.0	121.1	98.4
Alkaline p-nitrophenylphosphatase	191.9	103.8	153.5	76.5	217.8	160.2
γ-glutamyltranspeptidase	16.8	10.9	15.8	11.2	15.3	11.4

10^{10} lymphocytes in 200 ml Hepes buffered (pH 7.3) Dulbeccos's modified Eagles medium were stimulated for 6o min at 37° C with 10 µg/ml conA.

Taken together, the data demonstrate, that lymphocyte plasma membranes are not entirely homogeneous, but contain domains, consisting of the high-affinity mitogen receptor and some enzymes, e.g. (Ca^{2+}+Mg^{2+})ATPase, (Na^{+}+K^{+})ATPase and lysolecithin acyltransferase.

The assumption that there exist receptor-associated domains in the plasma membrane of lymphocytes bears the implication for the initiation of activation. When plasma membranes of conA-stimulated lymphocytes were separated by affinity-chromatography, the yield of the subfractions was similar to that of unstimulated ones (Rode et al., 1977, Resch et al., 1983). Upon stimulation with conA the specific activities of membrane bound enzymes were altered in a specific pattern. The enzymes which were randomly distributed in the plasma membrane, e.g. γ-glutamyl-transpeptidase, alkaline phosphatase and Mg^{2+}-ATPase were suppressed in their activities in a similar degree in both subfractions, whereas the enzymes predominantly located in the fraction containing the high-affinity mitogen receptor, e.g. (Na^{+}+K^{+})ATPase and lysolecithin acyltransferase, were activated, especially in the adherent fraction, MF_2 (Table 4).

As upon stimulation of intact lymphocytes with conA the plasma membrane receptors are reorganized to form "caps" the receptors must move in the plane of the membrane together with their associated enzymes. The close spatial association of (Na^{+}+K^{+})ATPase and lysolecithin acyltransferase lent support of the idea of their possible functional coupling.
This hypothesis was tested by the use of ouabain, the specific inhibitor of (Na^{+}+K^{+})ATPase.

Ouabain inhibited conA-stimulated RNA and DNA synthesis in a concentration dependent way. In human lymphocytes complete inhibition of macromolecular synthesis was measured at a concentration of 20 nM. An obvious possibility explaining the inhibitory effect of ouabain was the association of the inhibitory action of the glycoside with the functioning of the (Na^{+}+K^{+})ATPase, e.g. with the alteration of the active monovalent cation transport (Quastel and Kaplan, 1970). However, observations by us and others showed, that the inhibition of macromolecular synthesis occurred at ouabain concentrations nearly one order of magnitude lower than that affecting potassium transport and (Na^{+}+K^{+})ATPase activity (Segel et al., 1980, Szamel et al., 1980). These results suggested that the effect of ouabain is exerted rather via some other membrane-associated activities than the shift of monovalent cations by (Na^{+}+K^{+})ATPase.

Time kinetics of the inhibition of stimulated RNA synthesis showed a specific pattern. When ouabain was added to lymphocytes after increasing time intervals following conA stimulation complete inhibition occured only, if ouabain was added together or shortly after the mitogen. If the glycoside was

added later, RNA synthesis maintained the level it had reached by the time of glycoside administration. Similarly, RNA synthesis could be arrested at any time adding the hapten sugar α-methyl-mannoside. These results suggested that ouabain prevented lymphocyte stimulation by interfering with an early membrane associated event immediately depending on the binding of the mitogen (Szamel and Resch, 1981, Szamel et al., 1981). Strikingly similar results were obtained with a hydroxyprogesterone derivative, chlormadinone acetate. This drug was recently suggested to be a possible endogeneous digitalis-like substance (LaBella et al., 1979). Chlormadinone acetate inhibited conA-stimulated RNA synthesis dose dependently, and also in this case several times higher concentrations of the progestine were needed to inhibit the transport activity of $(Na^+ + K^+)$ATPase. The time kinetics of the effect of chlormadinone acetate was also nearly identical with that observed following ouabain or α-methyl-mannoside administration (Szamel et al., manuscript in preparation). These findings further substantiated the idea, that digitalis glycosides and digitalis-like substances interfere with an early membrane event during the initiation of lymphocyte activation.

One of the earliest events in activated lymphocytes is the increase in the turnover of plasma membrane phospholipids (cf. Ferber and Resch, 1976, see also accompanying contribution by Resch et al.), predominantly in their fatty acid moieties. Ouabain at the same dose range, suppressing the activation of macromolecular synthesis, also inhibited the incorporation of oleate into lecithin in stimulated human and rabbit lymphocytes (Szamel and Resch, 1981, Szamel et al., 1981) as well as in isolated plasma membranes (Table 5).

Table 5

Effect of ouabain on the incorporation of ^{14}C-oleate into lecithin of thymocyte plasma membranes

Preincubation of cells with ouabain	cpm x mg protein^{-1}			
	control		ouabain	
(hours)	control	conA	control	conA
1	5240	8585	n.d.	6293
4	3232	6653	3472	3903
20	2120	5296	2108	2471

Lymphocytes (5 x 10^7/ml) were preincubated with ouabain for the times indicated. After these incubation periods 10 µg/ml conA was added simultaneously with 10 nmol/ml ^{14}C-oleate and incubated for 1 h. Plasma membranes were prepared and phospholipids extracted as described by Szamel et al. (1981).

As we have already mentioned, both (Na^++K^+)ATPase and lysolecithin acyltransferase, the enzyme responsible for the transfer of long chain fatty acids in lymphocyte plasma membranes, have been found to be activated upon conA stimulation. After an hour of conA stimulation the activity of lysolecithin acyltransferase as well as that of the transport ATPase was about twofold compared to the control. Activation of both enzymes could completely be suppressed when lymphocytes were preincubated with ouabain before conA stimulation (Table 6). These data suggest, that the glycoside prevented the activation of plasma membrane phospholipid metabolism by interfering with the activation of lysolecithin acyltransferase.

The possibility, that the inhibition of the activation of lysolecithin acyltransferase and the following increase in the turnover rate of fatty acids of membrane phospholipids, due to an unknown side effect of ouabain, was precluded by the following findings. Increasing the concentration of extracellular potassium, which leads to the release of bound ouabain to its receptor, the inhibition of stimulated RNA synthesis as well as the inhibition of oleate incorporation proved to be completely reversible, showing, that the blockage of activation was mediated by the binding of ouabain to its receptor, e.g. to (Na^++K^+)ATPase (Szamel and Resch, 1981).

On the other hand the inhibitory action of the glycoside cannot be explained by changed ion pump activities, as i., at the concentrations of ouabain used for the inhibition of macromolecular synthesis and membrane phospholipid metabolism no changes in the cellular potassium content could be demonstrated. ii., the activation of membrane phospholipid metabolism was unaffected by changing extracellular potassium and sodium concentrations iii., the activity of lysolecithin acyltransferase proved to be unaffected by varying monovalent cation concentrations (Szamel and Resch, 1981, Szamel et al., 1981).

In agreement with earlier observations by us and others (Na^++K^+)ATPase was found to be activated in the plasma membrane of mitogen-stimulated lymphocytes (Averdunk and Lauf, 1975, Rode et al., 1979). As activation of (Na^++K^+)ATPase proved to be independent of protein synthesis, and associated with an increase in binding sites for ouabain, it is likely, that formely cryptic (Na^++K^+)ATPase sites become active following a conformational change in the plasma membrane induced by mitogens (Quastel and Kaplan, 1975). The putative conformational change of (Na^++K^+)ATPase is probably involved in the activation of lysolecithin acyltransferase (and in the preven-

Table 6

Effect of ouabain on the activity of membrane-bound enzymes in thymocyte plasma membranes

Preincubation with 5×10^{-6} M ouabain h		nmol x mg protein^{-1} x min^{-1}, 37° C			
		Mg^{2+}-ATPase	$(Na^{+}+K^{+})$ATPase	OleoylcoA: lysolecithin acyltransferase	ArachidonoylcoA: lysolecithin acyltransferase
1	control	22.06	8.90	2.07	3.86
	conA	15.24	11.70	3.54	8.39
	conA + ouabain	19.70	9.76	2.80	5.33
4	control	27.42	7.93	2.36	4.77
	control + ouabain	26.05	8.20	2.37	4.66
	conA	20.49	14.00	4.12	11.20
	conA + ouabain	27.29	7.80	3.28	7.74
20	control	37.65	15.68	2.28	4.25
	control + ouabain	36.90	16.10	2.33	3.85
	conA	30.68	22.30	3.19	7.43
	conA + ouabain	38.20	15.60	2.56	3.97

Rabbit thymocytes were suspended at a concentration of 5×10^{7}/ml in Dulbecco's modified Eagle's medium and incubated with or without ouabain for the times indicated. ConA (10 µg/ml) was added for a further incubation period of 1 h. Plasma membranes were prepared as described by Szamel et al. (1981).

tion of its activation by ouabain) as both enzymes are located in a close proximity within the membrane. Thus our findings are compatible with the notion that ouabain prevents activation of lysolecithin acyltransferase (and concomitantly, the activation of the phospholipid fatty acid turnover) by a direct functional coupling of the two enzymes in the plasma membrane domains of lymphocytes.

The finding, that digitalis glycosides and digitalis-like substances suppressed the early activation of the membrane phospholipid turnover strongly suggests, that activation of $(Na^+ + K^+)$ATPase and lysolecithin acyltransferase constitute essential steps of the triggering process itself. On the other hand, the results show that $(Na^+ + K^+)$ATPase is specifically implicated in the activation of cells by an interrelation of another enzyme of the plasma membrane of lymphocytes, constituting a part of the functional domains involved in the plasma membrane dependent control of cell growth and division.

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DISCUSSION

SCHONER:

In agreement with your data Erdman et al. have shown that concentrations of ouabain caused a positive inotropic effect in heart muscle without influencing the activity of (Na^+/K^+)ATPase.

SZAMEL:

Thank you for your comment, I know the data.

KEPES:

The effect of ouabaine at doses which do not inhibit Na-K ATPase seems difficult to explain at first sight, since inhibition is closely correlated with specific binding. However, ouabaine besides its inhibitory action has another effect, namely it enhances the phosphorylation of the E_2 form of the enzyme by inorganic orthophosphate. This E_2-P even in small proportion might have some catalytic effect, possibly via phosphorylation of some other key component of the system.

BHARGAVA:

Couldn't your inhibition of RNA and DNA synthesis - as measured by incorporation of an exogenous precursor - by ouabain, be due to inhibition of the transport of the precursor accross the cell membrane?

SZAMEL:

Interestingly, only incorporation of nucleotides and amino acids was influenced by ouabain, however, their transport was not inhibited at all.

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MITOCHONDRIAL COUPLING FACTOR B (F_B) AND THE F_o PROTON CHANNEL

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The terminal reaction in oxidative phosphorylation involves a reversible H^+ pump which drives the phosphorylation of ADP at the expense of the energy derived from the electrochemical H^+ gradient generated by respiratory reactions. The H^+ pump, which goes by a number of names including ATP synthase, H^+-ATPase and F_o-F_1 consists of a well-characterized hydrophilic segment and a hydrophobic membrane-associated segment (F_o). The analogous F_o of *E. coli* consists of three distinct subunits, but the mitochondrial F_o is more complex and has at least four distinct subunits (OSCP or oligomycin sensitivity conferring protein, F_6 or coupling factor 6, F_B and the dicyclohexylcarbodiimide (DCCD) and oligomycin binding proteolipid) (1).

Bovine heart mitochondrial F_B is a 14,600 dalton soluble protein essential for P_i-ATP exchange but not for the oligomycin sensitive ATPase activity (2,3). Cd^{2+} and phenylarsine oxide inhibit the coupling factor activity of F_B as well as the P_i-ATP exchange activity of the purified H^+-ATPase (4,5). The fact that a large excess of 2-mercaptoethanol does not block the inhibition but a 2 or 3-fold excess of a dithiol does, has led to the conclusion that F_B has a functionally important vicinal dithiol group. F_B is present in the H^+-ATPase in amounts stoichiometric with F_1 (unpublished data) and is associated with the F_o segment and not with F_1(3).

Two hypotheses have been presented to account for the stimulating effects of F_B on energy-linked reactions. One proposes that F_B is functional component of the H^+-ATPase and the other that removal of F_B produces damage to the membrane which results in increased H^+ conductivity and the $\Delta\mu H^+$ is discharged. We report here experiments to test which hypothesis holds.

For the determination of the exact role of F_B in ATP synthesis, two methods are available. One is the selective depletion of F_B by extraction with ammonia-EDTA and reconstitution of the depleted preparation with highly purified F_B. Second is inhibition of F_B by Cd^{2+} or phenylarsine oxide in the presence of excess 2-mercaptoethanaol. Demonstration of F_B as a part of the H^+-ATPase and more specifically of its F_o segment has also permitted sharper focus on the questions regarding its role. For the assay of the H^+-ATPase activity, besides the classical P_i-ATP exchange assay, we have followed the absorbance change of the voltage sensitive dye, oxonol VI (bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine

oxonol) in response to energization by ATP (6). For studying the role of F_B in the F_o, we have measured passive H^+ conduction mediated by a K^+ diffusion potential using quenching of 9-aminoacridine fluorescence as the assay (7).

Table I

ATP-driven H^+ uptake in submitochondrial particles: effect of F_B

	Uptake		ΔpH decay
	Maximum neq. H^+	Rate neg. H^+/min.	$t_{\frac{1}{2}}$ sec.
ETPH	30.1	6.6	38
AE-P	16.2	2.6	37
AE-P+F_B	21.5	5.2	31

The pH was monitored with a glass electrode in a medium consisting of 80 mM KCl, 1 mM glycylglycine, 2 mM $MgCl_2$, 1 mM Mops, 2 µg valinomycin and 1 mg of particle per ml. The F_B-deficient AE-P was saturated with F_B and excess removed by centrifugation. H^+ translocation was initiated by the addition of 80 µl of 5 mM ATP in 2 mM $MgCl_2$ (pH 5.2). The decay was measured following the addition of oligomycin (1 µg/ml) to block H^+ pumping. (From ref.6).

Table II

Effect of F_B and oligomycin on ATP-dependent oxonol binding.

	Binding		Decay
	Rate $\Delta\%T \times sec^{-1}$	Maximum ΔT	$t_{\frac{1}{2}}$ sec.
AE-P	0.58 (0.12)	2.15 (0.2)	16.0
AE-P+F_B	1.03 (0.01)	3.54 (0.2)	16.8
AE-P+ low oligo	1.08 (0.15)	5.9 (0.1)	

Oxonol VI (5 µl of 1-5 mM) was added to 200 µg particle in 3 ml 40 mM Tris-Cl (pH 7.4). Reaction was started with the addition of 2 µmol ATP and 0.2 µmol ADP. Decay rates were measured after the addition of oligomycin (1 µg/ml). Low oligomycin represents the concentration that maximally stimulated the activity of AE-P in ATP-driven NAD^+ reduction by succinate. Oxonol binding was measured as $\Delta T_{603-630}$ nm in a dual wavelength spectrophotometer. ( ), standard deviation (From ref.6).

When submitochondrial particles (SMP) were depleted of F_B, the deficient AE-particles showed a significant reduction in the rate and extent of H^+ uptake driven by ATP breakdown (Table I). Restoration of F_B increased both parameters. However, neither depletion of F_B nor its reconstitution affected the H^+ permeability of the membrane. In similar experiments, ATP-supported oxonol binding was measured as well as its discharge on addition of oligomycin (Table II). The binding was stimulated significantly both in magnitude and rate by the restoration of F_B to the deficient particle. The effect was similar to that of a low level of oligomycin, which is known to stimulate energy-linked reactions in these particles (8). However, the discharge rate was unaffected by F_B

restoration. Thus, experiments at the level of membrane complexity of SMP indicate that F_B does not bring about its stimulatory effects by blocking a H^+ leak.

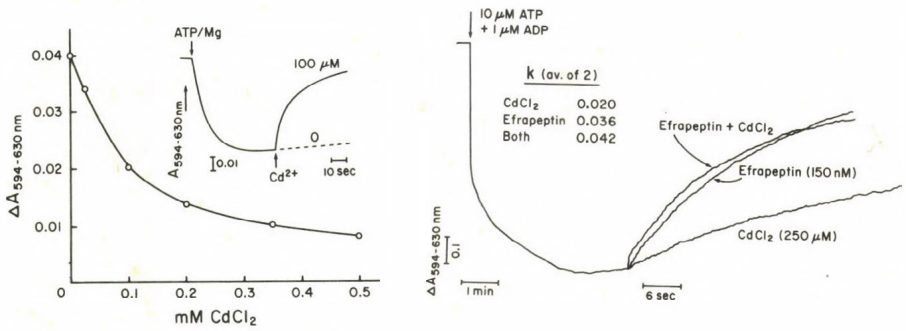


Fig.1 (left) Inhibition of ATP-energised oxonol VI binding in H^+-ATPase by Cd^{2+}.

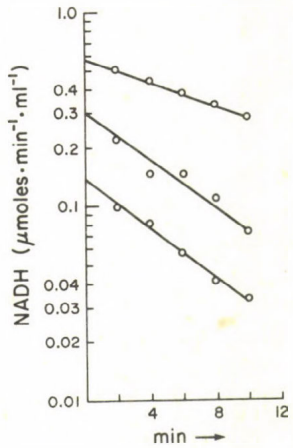
The reaction mixture consisted of 100 μg H^+-ATPase and 2 μl of 1.5 mM oxonol in 2 ml of 40 mM Tris acetate, pH 7.5, 0.25 M sucrose and 2 mM $MgCl_2$. ATP (10 μM) and ADP (1 μM) were added to start the reaction shown in the inset. Cd^{2+} (100 μM) was added after the membrane potential was generated. For the concentration curve, Cd^{2+} was added to the reaction mixture before ATP+ADP, and the maximum absorbance at steady state equilibrium was measured.

Fig.2 (right) Effect of Cd^{2+} on H^+ conductance rates.

The H^+-ATPase was energised with ATP in the presence of oxonol as in Fig.1. The efrapeptin and Cd^{2+} were added separately and together as shown on the traces. The first order rate constants for the absorbance discharge were derived from plots by logoa vs. time.

Now let us turn to experiments with the purified H^+-ATPase prepared by lysolecithin extraction of bovine heart SMP (9). Energization of H^+-ATPase in the presence of oxonol VI produces an absorbance change (Fig.1), the magnitude of which is dependent upon protein concentration. The absorbance change is reversed by valinomycin + K^+, oligomycin, uncouplers or Cd^{2+}. Mercaptoethanol was present in the experiments with Cd^{2+} to prevent its binding to protein monothiol groups. Presence of dithiothreitol (5:1), however, reduced the effectiveness of Cd^{2+}. Fig. 1 also shows a cadmium concentration curve for its effect on the steady state absorbance change. It may be noted that in the range shown the inhibition of H^+ pumping is not complete, and in the 100 to 200 μM level used in the subsequent experiment, there is still considerable activity. Thus, the decay on addition of Cd^{2+} seen in the insert is slower than the true rate of discharge, since recharging of the membrane potential with the residual ATP is still occurring under these conditions. In order to estimate the actual rate of discharge of membrane potential, it was, therefore, necessary to inhibit H^+ pumping, which was done with efrapeptin, a strong inhibitor of the

ATPase (10). At the concentration shown in Fig.2, efrapeptin inhbited the ATPase activity (P_i release in 10 min.) 98 and totally prevented the ATP-dependent absorbance change. Addition of efrapeptin to the H^+-lATPase after energization with a minimal amount of ATP, barely enough to give the maximal absorbance change, induced a discharge of the bound oxonol with a pseudo first order rate constant of 0.036. Cd^{2+} added together with efrapeptin produced no further significant increase in the decay rate, showing that in the H^+-ATPase also, Cd^{2+} inhibits H^+-pumping without increasing the H^+ conductance of the membrane.



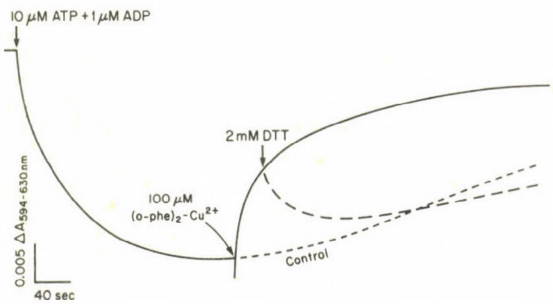


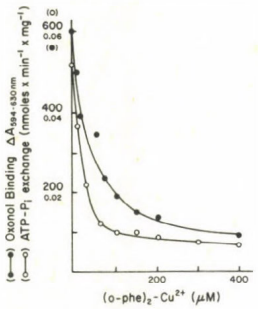
Fig.3 (left) Kinetics of F_B inactivation by copper o-phenanthroline.

F_B (0.33, 0.67 and 1.0 μM) was incubated with 80 μM $CuSO_4$ and 160 μM o-phenanthroline at 25° in 10 mM Tris-SO_4 pH 7.5 and 0.2 glycerol. Samples were removed every 2 min. and added to F_B-deficient AE-particles. The stimulation of the AE-particle activity in ATP-dependent NAD^+ reduction by succinate was measured (3).

Fig.4 (right) Discharge of oxonol bound to energised H^+-ATPase by copper o-phenanthroline.

H^+-ATPase was energised by ATP in the presence of oxonol VI was in Fig. 1. The copper chelate and dithiothreitol were added at the times shown on the trace.

If our conclusion that F_B contains a vicinal dithiol which binds Cd^{2+} and arsenicals is valid, then oxidation of the dithiol to disulfide might also inhibit F_B activity. In order to test the hypothesis, purified F_B was treated with copper o-phenanthroline (1:2) chelate, and iodosobenzoate separately and its acivity tested at intervals in the reversed electron flow assay (11). As seen in Fig.3, there is progressive inactivation of F_B, which follows pseudo first order kinetics, showing that the disulfide formation is an intramolecular reaction



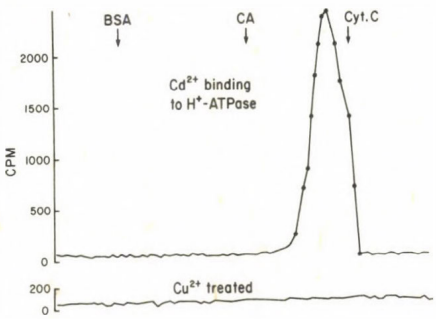


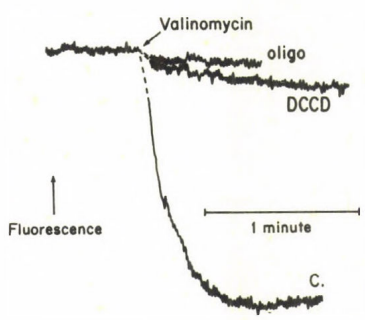
Fig.5 (left) Inhibition of P_i-ATP exchange and oxonol binding activities of H^+-ATPase by copper o-phenanthroline.

Inhibition of oxonol binding was determined as in Fig.1 for the effect on P_i-ATP exchange, 200 µg of H^+-ATPase was added to 100 µmol of Tris-SO_4, pH 8.0, containing 0.5 µmol $MgCl_2$, 2.5 mg bovine serum albumin, $3x10^5$ cpm ^{32}P-phosphate and different amounts of copper o-phenanthroline on 0.69 ml. The reaction was carried out 38° for 15 mins. after addition 15 µmol ATP, 5 µmol ADP and 20 µmol $MgCl_2$, as described previously (3).

Fig.6 (right) Binding of $^{115}Cd^{2+}$ to F_B- H^+-ATPase.

H^+-ATPase (20 mg/ml) was incubated with or without 50 µM copper o-phenanthroline in 10 mM Tris SO_4 pH 7.5 and 0.25 M sucrose for 10 min. at room temperature, the samples were chilled and the protein was recovered by centrifugation. The H^+-ATPase was resuspended and $^{115}CdCl_2$ was added to 125 µM. After incubation for 5 minutes, the samples were brought to 1 SDS and 5 glycerol, and 50 µg protein was electrophoresed. Gel slices were extracted for 48 hours with 1 ml of 20 mM EDTA for counting.

Iodosobenzoate gave results quite similar to those with copper o-phenanthroline. These two reagents are considered highly specific thiol oxidants (12,13). We had previously reported that ^{115}Cd added to H^+-ATPase at concentrations that inhibited P_i-ATP exchange activity was recovered at a position corresponding F_B in SDS-PAGE (2). If the H^+-ATPase is first treated with copper o-phenanthroline in order to oxidize the dithiol to disulfide, $^{115}Cd^{2+}$ binding is completely abolished (Fig.6). These results show with a fair degree of certainty the presence of a vicinal dithiol in F_B and that Cd^{2+} is chelated to the dithiol. Furthermore, the inhibitory action of Cd^{2+} on H^+-ATPase is most probably caused by its binding to F_B.



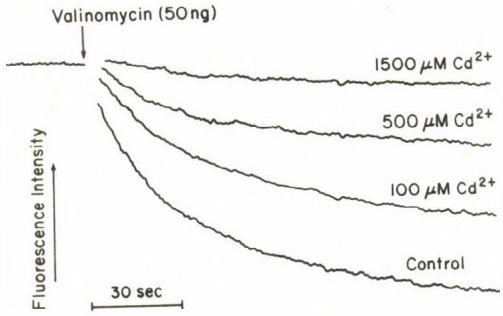


Fig.7 (left) Passive H^+ conduction by proteoliposomes containing F_o.

Twenty mg of asolectin dried in a thin layer were suspended in 1 ml of 200 mM KCl, 20 mM Tricine buffer, pH 7.5, shaken and dispersed by mild sonic treatment. One mg of F_o pellet from a routine NaBr treatment of H^+-ATPase was suspended with the liposome preparation. The mixture was frozen and thawed, sonicated briefly and passed through Biogel P-6 DG column equilibrated with 200 mM NaCl and 10 μM Tricine buffer. Small aliquots of K^+-loaded liposomes were assayed in 2 ml containing 5 μM 9-aminoacridine. Valinomycin (50ng) was added and fluorescence was monitored to measure H^+ flow through the H^+ channel. The excitation and emission wavelengths were 365 nm and 451 nm respectively. Where shown -10 μM DCCD and -5 μg oligomycin were added one minute before addition of valinomycin.

Fig.8 (right) Inhibition of passive H^+ conduction through F_o by Cd^{2+}.

The experiment was carried out as in Fig.7. Cd^{2+} was added prior to valinomycin.

Since F_B is a component of F_o, a more direct method of examing its role would involve a study of F_o activity measured by passive H^+ conduction. Proteoliposomes containing F_o were loaded with K^+, the external K^+ was removed by gel filtration and assayed in a NaCl medium. The quenching of 9-aminoacridine fluorescence was monitored following the addition of valinomycin to initiate the K^+ diffusion potential. Oligomycin and DCCD inhibited the fluorescence quenching completely (Fig.7). Similarly, in the presence of Cd^{2+} also, the fluorescence quenching was inhibited (Fig.8).

We have shown previously that routine F_o preparations have P_i-ATP exchange or ATPase activities, and both are restored partially by the addition of coupling factor 1 (F_1) (3). On the other hand, F_o preparations exhaustively depleted of F_B are capable of rebinding F_1 with restoration of OSATPase activity but not of P_i-ATP exchange activity and F_1 does

not contain any F_B, it would follow that routine preparations of F_o, as isolated by NaBr treatment of H^+-ATPase contain F_B. Figure 9 shows that such F_o binds $^{115}Cd^{2+}$ and the protein bound cadmium is recovered at the position of F_B in SDS-PAGE. The ^{115}Cd recovered in the band co-migrating with F_B amounted to 0.73 moles per mole of F_o, assuming a molecular weight of 150,000 for the F_o complex.

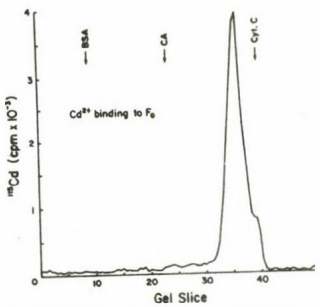


Fig.9 Labeling of F_B in F_o by $^{115}Cd^{2+}$.

One mg F_o in Tris acetate pH 7.5 containing 250 mM sucrose and 1 mM 2-mercaptoethanol were incubated for 5 minutes with 25 nmol $CdCl_2$ (0.5 μc_1/nmole). SDS-PAGE was carried out on aliquots containing 50 µg protein.

These results showing F_B involvement in H^+ conduction by F_o seem inconsistent with several reports indicating H^+ conductance mediated by the purified DCCD binding proteolipid alone in liposomes (14,15) and lipid-impregnated millipore membranes (16). Schindler and Nelson (17) have reported for the first time discrete single conductance channels with a highly purified preparation of the proteolipid from yeast mitochondria. The reasons for the discrepancy need to be determined. However, our data that F_B is a necessary part of the F_o H^+ channel are consistent with the results using F_o derived from E. Coli unc mutants, showing requirement of more than one F_o subunit (18-20), in fact all three (21) for H^+ conduction. It is yet to be determined whether F_B dithiol is functional in actual H^+ conduction by participating in a series of protonation-deprotonation reactions or is necessary for maintaining a fully functional H^+ channel.

Acknowledgements

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DISCUSSION

ERNSTER:

What is the relationship of mitochondrial Factor B to the 3 subunits of E.coli F_0?

SANADI:

We do not know, but some properties e.g., case of extraction and high polarity suggest a similarity to the b subunit.

MONTECUCCO:

Do you have any structural information on your factor B?

SANADI:

We know only the amino acid composition. N-terminal amino acid sequence determination is in progress.

FONYÓ:

It appeared from your figures that in different systems shown you required different concentrations of Cd^{2+} for inhibition and I was somewhat astonished to see that in your last system shown 1.5 mM Cd^{2+} was the concentration that gave full inhibition. Would you care to comment on this?

SANADI:

It is correct that as you purify the preparation, the I_{50} value increases substantially. Also there is a difference in Cd^{2+} concentration necessary for inhibition depending on the source of mitochondria. Heart systems are far less sensitive than liver mitochondria and its derivative preparations. There is analogy for these observations with other inhibitors, e.g., oligomycin.

TIGYI:

I was pleased to hear your results concerning the effect of Cd^{2+} and Cu^{2+} on proton transfer system. During the

recent years we studied a completely different problem: the radiosensitivity and primary effects of ionizing radiation using the scintillation effect of tritium β particles in muscle.
We found similar effects of Cd and Cu. It seems to be an analogy and it is possible that both phenomena have common molecular basis.

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SOME ASPECTS OF THE HUMAN RED CELL CALCIUM PUMP

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INTRODUCTION

In the last 10 years evidence has accumulated to the effect that in general animal cells rely on a dual mechanism for keeping intracellular Ca^{2+} concentration low.

I. In the plasma membrane there is a Ca-Na-exchange system that extrudes 1 Ca^{2+}-ion uphill in exchange for 3 (or more) Na^{+}-ions per cycle. This system is not energized directly but normally exploits the energy stored in the Na-gradient built up by the Na-K-pump. The system is not directional (it is equally possible that a Ca-gradient moves Na) and in addition exchanges Ca^{2+} for Ca^{2+}. Its existence has the consequence that the Na-gradient and the Ca-gradient across the membrane are not independent of each other but, provided that the ratio of affinity (K_{Na} / K_{Ca}) is equal on both sides of the membrane, obey the equation

$$\frac{[Ca_i^{2+}]}{[Ca_o^{2+}]} = \frac{[Na_i^{+}]^n}{[Na_o^{+}]^n} \cdot \exp\left[(n-2) \cdot \frac{E_m F}{RT}\right] \quad (1)$$

where subscripts designate free concentrations inside and outside the cell and n is the number of Na^{+} ions transported in exchange for 1 Ca^{2+}-ion. The exponential term takes into account the membrane potential (E_m) which matters when n is assumed to be 3 or 4, rendering the system electrogenic.

II. The second Ca^{2+} exporting system in the plasma membrane is an ATP-fuelled Ca-pump. The coupling of Ca-movement with Na-movement across the Na-Ca-exchange system forces the Ca-pump to contribute to the Na extrusion whenever conditions are such that the Ca-pump tends to lower the intracellular Ca^{2+} concentration to below that given by the Na-gradient (caused by the Na-K-pump) according to equation (1). The human red cell membrane is devoid of the Na-Ca-exchange system and therefore well suited for the study of the Ca-pump and in point of fact was the first cell in which the existence of the pump was demonstrated (Schatzmann, 1966).

The intracellular free Ca^{2+} concentration in red cells is below 10^{-7} M (3×10^{-8} M) (Lew et al., 1982) owing to the rather low

passive permeability of the red cell membrane for Ca^{2+} (50 µmoles/l·h under physiological conditions) (Lew et al., 1982) and the effective active outward Ca^{2+} transport (v_{max} at saturating Ca^{2+} concentration being some 10 mmoles/l·h). If the figure for internal Ca^{2+} concentration is correct, it appears thermodynamically impossible that the stoichiometric ratio of the process (moles of Ca^{2+} transported per mole of ATP hydrolysed) is higher than 1 (Schatzmann & Roelofsen, 1977; Clark & Carafoli, 1983; Muallem & Karlish, 1979). Yet there is good evidence for more than 1 Ca^{2+}-ion being involved (Ferreira & Lew, 1976). This might mean that a second Ca^{2+}-ion which is not transported is required for activation of the system.
It has been shown (Waisman et al., 1981a, 1981b; Rossi & Schatzmann, 1982a) that Ca^{2+} is not exchanged for another metal cation across the pump. In particular, Mg^{2+} can be excluded as a counterion. By the same vein it was demonstrated that Ca^{2+} is not accompanied by Cl^- on its way through the pump mechanism (Rossi & Schatzmann, 1982a). Therefore, the pump is either electrogenic or exchanges Ca^{2+} for protons (Niggli et al., 1982).
If a comparison is made of the transport in the presence of choline or tris as major cation with that of cells in the presence of Na or K it is found that both Na and K have a stimulatory effect (30 - 50 %) which is clearly elicited on the inside of the membrane (Kratje et al., 1983). The biological significance of this behaviour is obscure at the present time since the affinity for K is about 5 times higher than that for Na in this effect.
The system is an "ATPase" which depends on Ca^{2+} and Mg^{2+}. In disrupted membranes its affinity for Ca^{2+} increases with pH; K_{Ca} was found to be 1.4 µM at pH 6.7 and 0.2 µM at pH 7.8 (Luterbacher, unpublished). K_{Mg} (for the free ion) is 14 - 35 µM. An important point, which will be made clear below, is that the activation by ATP is biphasic. The enzymic site has a $K_m \sim$ 3 µM for total ATP, whereas a modifier site appears to have a dissociation constant of 80 - 300 µM (Richards et al., 1978; Muallem & Karlish, 1979, 1980, 1981; Stieger & Luterbacher, 1981). An alternative way of understanding this behaviour is to assume one ATP binding site whose affinity is low in the E_2 configuration and high in the E_1 configuration (see fig. 1). This assumption, however, leads to the hypothesis of a dimeric functional unit with "half-of-the-sites" reactivity.

THE REACTION CYCLE

The splitting of the terminal P-O-P bond of ATP proceeds under transient phosphorylation of the protein which can be studied using $[\gamma^{32}P]$-ATP (see fig. 1).

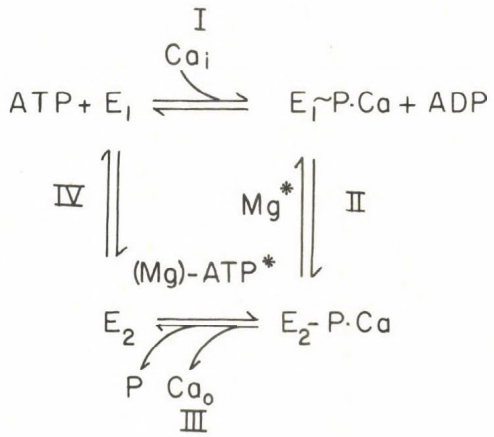

Fig. 1: Reaction cycle of the human red cell Ca-pump. The cycle is started by phosphorylation of the enzyme from ATP in the E_1 configuration under the influence of internal Ca^{2+}. The cycle moves subsequently in clockwise direction. Asterisks mark the requirements of the reaction. ATP means high concentration ($\geq$ 100 µM). P means inorganic phosphate. The assumption that Ca^{2+} is released outside simultaneously with dephosphorylation (inside) is hypothetical.*

Phosphorylation occurs at an acyl group (Lichtner & Wolf, 1980) and requires the presence of Ca^{2+}. Two different lines of evidence suggest that the phosphorylated protein ($E_1 \sim P$) undergoes a conformational change ($E_1 \sim P \rightarrow E_2 - P$) which requires the presence of Mg^{2+}-ions (Garrahan & Rega, 1978; Luterbacher & Schatzmann, 1983). Since Ca^{2+} stimulates ATP splitting by passing through the system from inside to outside E_1 clearly is the conformation in which the Ca^{2+} binding site faces the internal membrane surface. It is of course likely that the $E_1 \sim P \rightarrow E_2 - P$ conformational change is identical with the intramolecular motion required to bring Ca^{2+} from inside to the outside of the membrane. Dephosphorylation is accelerated by high ATP concentration ($\geq$ 100 µM) (Garrahan & Rega, 1978) which is an explanation for the low affinity site seen in the overall ATPase reaction.
So far the reaction cycle has defied detailed analysis. In the steady state only a small fraction of the protein ($\sim$ 10 %) is in the phosphorylated form (Luterbacher, 1982). The fact that with or without a Ca^{2+}-gradient from outside to inside it is not feasible to incorporate measurable amounts of inorganic phosphate ($^{32}P_i$) into the protein points to the possibility that the dephosphorylation reaction (III) is strongly poised towards E_2 (Luterbacher, 1982). From experiments with La^{3+} (see below) we have some approximate notion about the position of the equilibrium of reaction I. This step seems to be endergonic which means that dephosphorylation must be strongly exergonic (Luterbacher, 1982).

CALMODULIN DEPENDENCE

Unlike the sarcoplasmic reticulum Ca^{2+}-pump the red cell Ca^{2+} transport system is stimulated by calmodulin (CaM) which is present in the cytosol at 3 - 5 µM concentration (Foder & Scharff, 1981). The activator protein originally found by Bond an Clough (1973) was shown to be calmodulin (Larsen & Vincenzi, 1979). As in other CaM-dependent systems calmodulin binds to the pump when associated with Ca^{2+} (at 3 or 4 of the 4 metal binding sites). The effect of CaM binding is that the affinity of the transport site for Ca^{2+} increases about 30-fold, and the v_{max} rises somewhat (Scharff & Foder, 1978). Interestingly, the stimulation by monovalent cations becomes more prominent in the calmodulin-associated state (Scharff, 1978). It must be stressed that without CaM there is definitely some basal activity and that under artificial experimental conditions (see below) full activity and high Ca^{2+} affinity are displayed without CaM. Thus the pump protein contains all necessary constituents for Ca^{2+} transport and Ca^{2+} associated with CaM is not the Ca^{2+} transported.

Scharff and Foder (1982) have elegantly dissected the process of pump-CaM association. In their experiments the dissociation constant increased from 2.5 nM to 25 µM when the Ca^{2+} concentration was reduced from 20 µM to 0.1 µM. Therefore, at the physiological Ca^{2+} concentrations in red cells ($\leq$ 0.1 µM) most of the pump protein will be free of CaM. With rising Ca^{2+} concentration the rate constant of the pump-CaM dissociation reaches its minimum (0.044 min^{-1}) at 1 µM Ca^{2+} and the rate constant for association becomes maximal at 10 µM Ca^{2+} (17.3 x 10^6 $M^{-1}min^{-1}$) (Scharff & Foder, 1982). This behaviour and the low rates result in a hysteretic behaviour with respect to Ca^{2+} concentration changes. A sudden increase in the cell permeability to Ca^{2+} can be predicted to cause the Ca^{2+} concentration to pass through a transiently high concentration before reaching the new steady state. This behaviour was indeed found by Ferreira and Lew (1976) and Scharff et al. (1983).

In the presence of acidic lipids CaM is not required for high Ca^{2+} affinity and full activity of the system (Niggli et al., 1981a, 1981b; Stieger & Luterbacher, 1981). Even more interesting is the fact that controlled trypsin digestion (for a few minutes at $\sim$ 0.5 µg/ml and 37° C) dispenses with the calmodulin requirement, too (Taverna & Hanahan, 1980; Sarkadi et al., 1980, Niggli et al., 1981b; Stieger & Schatzmann, 1981) and removes the binding site for CaM (Enyedi et al., 1980). This proteolytic activation of the CaM free enzyme is achieved only in the presence of micromolar Ca^{2+} concentrations (Rossi & Schatzmann, 1982b). More resolute trypsinization in the absence of Ca^{2+} destroys the excess activity elicited by CaM (Rossi & Schatzmann, 1982b). This may mean that in the absence of Ca^{2+} trypsin removes the CaM receptor site while in the presence of Ca^{2+} (at the transport site) an additional sequence is cleaved off which is inhibitory without CaM and from whose inhibitory action the system is released by CaM binding.

PURIFICATION OF THE PUMP PROTEIN

The protein of the pump has been purified to near homogeneity on CaM affinity columns (Niggli et al., 1979; Gietzen et al., 1980). It has a monomeric mol.weight of 140'000 and survives for some hours when incorporated into phospholipid micelles on the column. This purified material has all the properties of the membrane-bound (Ca^{2+} + Mg^{2+})-ATPase and can be made to transport Ca^{2+} uphill when incorporated into artificial lipid vesicles (Carafoli & Niggli, 1981; Stieger, 1982). It is unknown whether it is active as a monomer or whether a dimer or oligomer is present in the membrane and is formed upon reconstitution. Indirect evidence suggests the existence of at least a dimeric structure (Hinds et al., 1982).

INHIBITORS

No specific inhibitory agent has been found yet. Lanthanum (La^{3+}) blocks the system at 0.1 mM completely. In its presence the amount of phosphoprotein formed exceeds by far the steady state amount reached in the unpoisoned system. The phosphoprotein is in a form that can be backreacted with ADP but not dephosphorylated in the foreward direction (Szasz et al., 1978; Schatzmann & Bürgin, 1978; Luterbacher & Schatzmann, 1983). Thus $E_1 \sim P$ seems to accumulate in La^{3+} poisoning which means that La^{3+} blocks the $E_1 \sim P \rightarrow E_2 - P$ transition. Vanadate ($H_2VO_4^-$) blocks the overall reaction nearly completely at micromolar concentrations if 4 mM Mg^{2+} and 120 mM K are present and the ATP concentration is low (Barrabin et al., 1980; Rossi et al., 1981; Stieger, 1982). However, a saturating vanadate concentration reduces the steady state phosphoenzyme only by 40 - 50 % (Luterbacher, 1982). If the protein is phosphorylated for minutes with unlabelled ATP in the presence of vanadate a subsequent test with $[\gamma^{32}P]$-ATP gives low ^{32}P-phosphoprotein values that correspond to the degree of inhibition of the overall (ATPase) reaction (Luterbacher, 1982). This can be interpreted by assuming that vanadate arrests the enzyme in the E_2-form which is plausible because at pH 7 vanadate is present mostly as $H_2VO_4^-$ resembling $H_2PO_4^-$ and, therefore, might fit the phosphate site of the system which appears on the E_2-configuration.

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DISCUSSION

SOMOGYI:

Dr. Martonosi mentioned some idea about the role of vanadate in the crystallization process of SR-Ca-ATPase and you named some other points of action for vanadate. Could you explain these differences including the different sensitivity of Ca-Mg-ATPase against vanadate in SR and in plasma membrane?

SCHATZMANN:

I have understood that Dr. Martonosi's success is due to the dodge of transforming all the conformational forms of the ATPase that coexist normally into one species, namely the vanadate-E_2-complex. We think that the blocking of the function is caused by the arrest of the reaction cycle in the E_2-form combined with vanadate. Thus there is not any difference in the views.

MARTONOSI:

What is the effect of Ca^{2+} on the vanadate induced inhibition of Ca^{2+} ATPase in erythrocytes?

SCHATZMANN:

Ca^{2+} antagonizes the vanadate effect. According to Rossi et al. this Ca^{2+} action seems to be exerted from the external side of the membrane.

MARTONOSI:

0.6 M KCl interferes with the vanadate induced crystallization of Ca^{2+}-ATPase. This is not necessarily attributable to a K^+ effect on the conformational equilibrium of Ca^{2+}-ATPase since other effects of K^+ (for example, on net surface charge) could also come into consideration.

SCHATZMANN:

I agree.

KÖVÉR:

Have you used intact red cells, or leaky cells or ghosts in your experiments? Have you applied vanadate in external medium affecting the membrane from the external surface, not from the cytoplasmic surface as in the case of SR, have not you?

SCHATZMANN:

The experiments shown were done on white membranes made leaky by freezing-thawing. It is true that the point of attack of vanadate is on the internal membrane surface. In intact red cells, however, it has an effect, because vanadate penetrates across the membrane.

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ATP-ANALOGUES AS A TOOL FOR THE STUDY OF $(Na^+ + K^+)$-ATPase

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INTRODUCTION

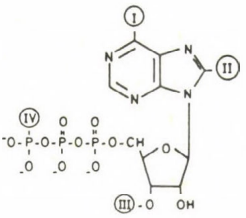
$(Na^+ + K^+)$-ATPase - the biochemical equivalent of the sodium pump of animal cell membranes - is electrogenic since it pumps 3 Na^+ ions out of the cell and 2 K^+ ions into it per ATP hydrolyzed (1). The enzyme protein consists of α- (M_r=100 000) and β- (M_r=30-50 000) subunits forming an αβ dimer. It is unknown so far, whether the αβ dimer or its multiples represent the pumping unit of the cell membrane (2). The subunit, which contains the ATP binding site, the acceptor site for a phosphorylated intermediate and the binding site for the specific inhibitor, the cardiac glycosides, exists in 2 conformational states called E_1 and E_2 (2). These conformational states have been demonstrated by the different reactivities of 2 forms of the phosphointermediate towards ATP and K^+ (3, 4), by their different sensitivities towards trypsin (5, 6) and via the tryptophan fluorescence (7). These studies furthermore showed, that $(Na^+ + K^+)$-ATPase exists in a Na^+ and a K^+ form (2). These forms are probably involved in the $Na^+ + K^+$ cation transport process which appears to proceed via ion gated channels (8) formed by ionophoric activity (9, 10).

Cation transport through $(Na^+ + K^+)$-ATPase is a rather quick process with a turnover number of 5 000 moles $P_i \cdot min^{-1} \cdot \alpha$ subunit^{-1} (11). The molecular events of the transport process may much easier be analyzed when the turnover can be turned down. Since Grisham (12) has already shown that Na^+ and K^+ enter the

ATP binding site, one has consequently to postulate, that the Na^+-transport starts and the K^+-transport ends there (13). From these studies the molecular structure of the ATP binding site receives considerable attention, because the ionophoric structures appear to be attached to the ATP binding site. To slow down the turnover rate of $(Na^+ + K^+)$-ATPase and to characterize the molecular structure of the ATP binding site as well as the effects of the cation substrates and effectors on it, ATP analogues were used to elucidate mechanisms of the sodium pump.

EVALUATION OF THE MOLECULAR STRUCTURE OF $(Na^+ + K^+)$-ATPase AND OF THE ATP BINDING SITE BY ATP ANALOGUES

Despite the earlier report that the sodium pump of human red blood cells is ATP specific (14) it is possible to demonstrate that purified $(Na^+ + K^+)$-ATPase recognizes a variety of substituted and altered ATP molecules (Fig. 1). Photoaffinity labelling of $(Na^+ + K^+)$-ATPase with α-^{32}P labelled derivatives of 8-azido-ATP (8-N_3-ATP) or 3'-O-(3-/2-nitro-4-azidophenyl/-propionyl)ATP (3'-N_3-ATP) leads to the incorporation of radioactivity into the α-subunit of M_r=100 000 (15, 16) (Fig. 2). Thus it was possible for the first time to demonstrate with the purified enzyme that the earlier assumptions were justified i.e. the α-subunit may contain the ATP binding site (11, 17). Although ATP photoaffinity labels are suitable to localize the ATP binding site on one of the subunits of the $\alpha\beta$ dimer, it is not possible to titrate the number of ATP binding sites per mole of enzyme with them and to contribute thereby to the discussion whether the $(Na^+ + K^+)$-pump may work as a monomer (2), dimer (11, 17) or tetramer (18) of $\alpha\beta$-subunits. The titration of active sites of $(Na^+ + K^+)$-ATPase needs the availability of protein reactive ATP analogues e.g. the S-(2,4-dinitrophenyl)--6-mercaptopurine triphosphate (dnp-sITP) (Fig. 1) which has been shown to react with a sulfhydryl group within the ATP binding site (19). This SH group opposed to the 6-amino group of purine ring is probably involved in the recognition of ATP, which is bound stronger and hydrolyzed faster than ITP (19, 28).



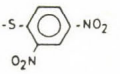
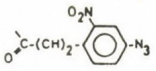
	K_D [M]	Temp. [°C]	Substituents in position I	II	III	IV	tissue source of $(Na^+ + K^+)$-ATPase
ATP	1.8×10^{-7}	0	$-NH_2$	H	H	O	beef brain
	$3 - 6.5 \times 10^{-6}$	37	$-NH_2$	H	H	O	pig kidney and beef brain
CrATP	4.3×10^{-5}	37	$-NH_2$	H	H	O	pig kidney
dnp-sITP	5.4×10^{-6}	0		H	H	O	beef brain
Cl-ITP	9.2×10^{-6}	0	-Cl	H	H	O	beef brain
sITP	1.8×10^{-5}	0	-SH	H	H	O	beef brain
ITP	5.8×10^{-5}	0	-OH	H	H	O	beef brain
$(sITP)_2$	1.8×10^{-5}	37	-S-	H	H	O	pig kidney
8-Br-ATP	4.2×10^{-6}	0	$-NH_2$	Br	H	O	beef brain
8-N_3-ATP	2.3×10^{-7}	0	$-NH_2$	N_3	H	O	pig kidney
3'-N_3-ATP	1.9×10^{-5}	21	$-NH_2$	H		O	pig kidney
ATPγS	2.2×10^{-7}	0	$-NH_2$	H	H	S	beef brain
ATPγF	2.4×10^{-6}	0	$-NH_2$	H	H	F	beef brain

Fig. 1: Structure-affinity relationship of modified ATP with $(Na^+ + K^+)$-ATPase (from refs. 16, 40).

An ATP analogue which reacts considerably faster is the disulfide of thioinosine triphosphate ($(sITP)_2$, $(SnoPPP)_2$) (20). This binds also with a higher affinity to $(Na^+ + K^+)$-ATPase than the dinitrophenyl derivative of sITP does (Fig. 1). $(sITP)_2$ inactivates $(Na^+ + K^+)$-ATPase by a disulfide exchange reaction (20, 21). As it is evident from Fig. 3 inactivation of $(Na^+ + K^+)$-ATPase by the disulfide of thioinosine triphosphate proceeds with two different reaction velocity constants. ATP protects the enzyme completely against the inactivation. Kinetic experi-

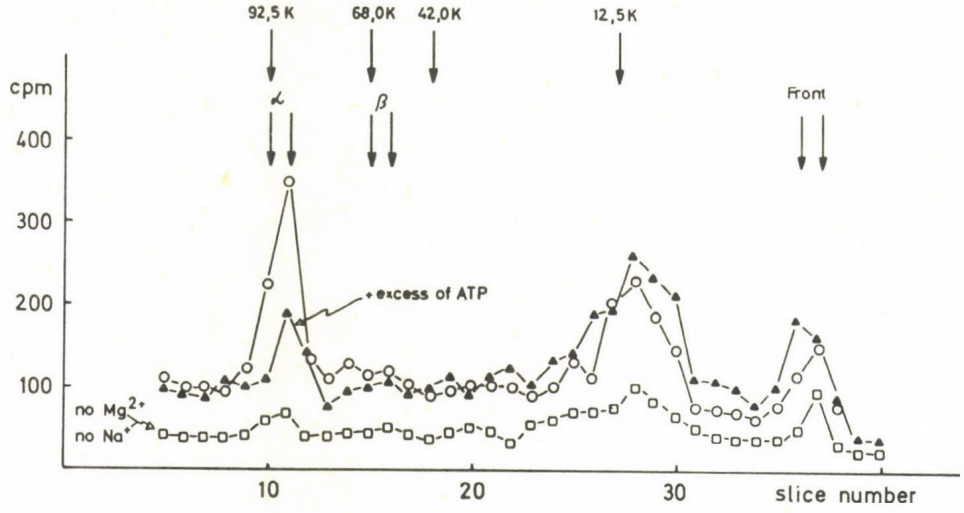


Fig. 2: SDS-electrophoresis of $(Na^+ + K^+)$-ATPase from pig kidney after photoaffinity labelling with 3'-O-(3-/2-nitro-4-azidophenyl/-propionyl)-(α-^{32}P)ATP (from ref. 15).

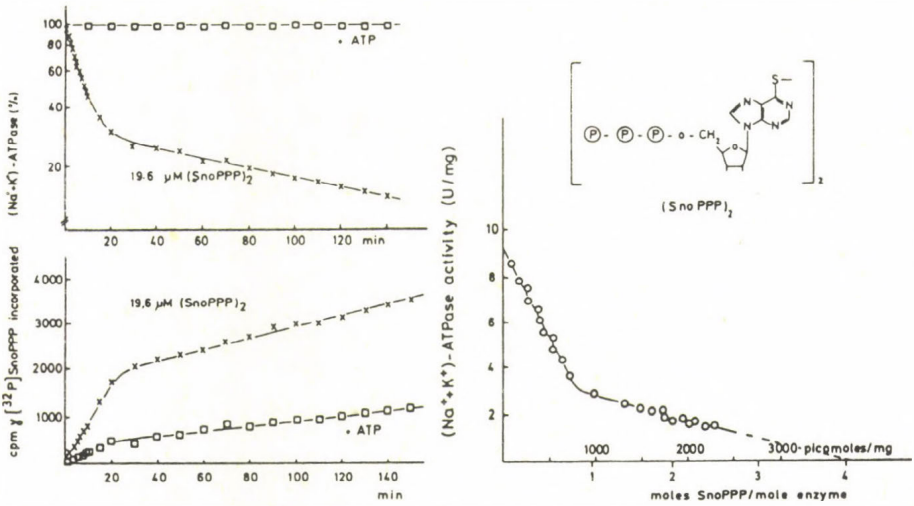


Fig. 3: Titration of the capacity of ATP binding sites of $(Na^+ + K^+)$-ATPase reacting with 21 µM $(/\gamma\text{-}^{32}P/SnoPPP)_2$. A) Inactivation of the enzyme; B) Incorporation of radioactivity into the enzyme protein; C) Plot of the activity remaining versus the incorporation of radioactive SnoPPP (from ref. 21).

ments demonstrate that $(sITP)_2$ binds with a dissociation constant of 18 μM to both sites bearing these SH groups. From protection experiments with ATP, however, it is evident that the rapidly reacting SH group belongs to a high affinity ATP binding site with a dissociation constant of 2.95 μM while the slowly reacting SH group refers to a low affinity ATP binding site with a dissociation constant of 77 μM (21). Inactivation experiments with the radioactive ATP analogue demonstrate that the capacity of the rapidly reacting SH group is equivalent to the capacity of the phospho-intermediate or the ouabain binding site and is thus equivalent to 1 mole of the α-subunit. 3 additional SH groups must be labelled to achieve complete inactivation (Fig. 3). Since excessive trypsinolysis of the radioactive product led to the demonstration of one single peptide only in a fingerprint analysis, it is plausible to assume that the differing reactivities of the SH groups in the α-subunits are due to the existence of 2 conformations of the enzyme showing high and low affinity ATP binding sites. The existence of high and low affinity ATP binding sites has also been demonstrated by others (17). It is suggestive to assume that this behaviour is induced by protein protein interactions of the α-subunits of the tetrameric enzyme (21).

SUICIDE OF $(Na^+ + K^+)$-ATPase WITH THE MgATP COMPLEX ANALOGUE CHROMIUM(III)ATP

Structural data do not provide all the necessary information to understand, how the active cation transport proceeds through $(Na^+ + K^+)$-ATPase. Additional kinetic experiments are necessary to get this understanding. Since ATP hydrolysis and the cation transport proceed with a considerable velocity, it was desirable to find means to slow down the turnover of the enzyme. Such a decrease in the velocity of the overall reaction might be possible by the use of the stable MgATP complex analogue chromium(III)ATP (CrATP) (22) which has been shown to slow down the turnover of kinases into a more convenient time scale (23). This ATP analogue seemed particularly interesting because Mg^{2+} ions appear to have a special function in the

overall process of the enzyme: Although Mg^{2+} ions are not necessary for the recognition of ATP (24-26), they are apparently needed as MgATP for the formation of a phosphorylated intermediate, whose maintenance in the reactive state appears to be governed by divalent cations (27). At least one divalent cation appears to be located at the active site, as it is apparent from nuclear magnetic resonance studies (12, 13). It is a matter of debate whether free Mg^{2+} ions are additionally necessary for the conversion of the E_1 to the E_2 conformational state within the transport cycle (30-32). CrATP could be a means to see whether free Mg^{2+} affects the enzyme. Moreover, CrATP has been reported to inactivate Ca^{2+}-ATPase of sarcoplasmic reticulum and to occlude Ca^{2+} within this enzyme in a stable form (33). Since Ca^{2+}-ATPase shows similar properties as $(Na^+ + K^+)$-ATPase (34), we considered it useful to test if CrATP is a substrate of $(Na^+ + K^+)$-ATPase.

In agreement with the assumed stability of CrATP as a MgATP complex analogue, CrATP could not substitute ATP as energy source to drive the cation transport in a reconstituted $Na^+ + K^+$-pump in liposomes containing purified $(Na^+ + K^+)$-ATPase from rabbit kidney. It behaved like the substrates of a partial reaction of the enzyme the K^+-dependent phosphatase (17). Their substrates could not fuel the active cation transport either (Fig. 4). Nevertheless, CrATP inactivated $(Na^+ + K^+)$-ATPase in the absence of Mg^{2+} and univalent cations (Fig. 5A). The apparent affinity was 43 μM at 37°C, whereas a dissociation constant of 8 μM was found for the β,γ-bidentate of CrATP (37-39). This finding suggests that Mg^{2+} is bound in the MgATP complex serving as the true substrate of $(Na^+ + K^+)$-ATPase (31) between the β and the γ phosphori. Apparently, the $(Na^+ + K^+)$-ATPase needs a straight triphosphate chain in the ATP binding sites for its function and not a curved one (which is formed by the α, β, γ-tridentate of CrATP). The inactivation by CrATP is hindered by the additional presence of K^+ (Fig. 5B). The site recognizing CrATP shows therefore the properties of the ATP binding site of $(Na^+ + K^+)$-ATPase, since this site is also incapable of binding ATP in the presence of K^+ (24, 25). Na^+ enhances, however, the

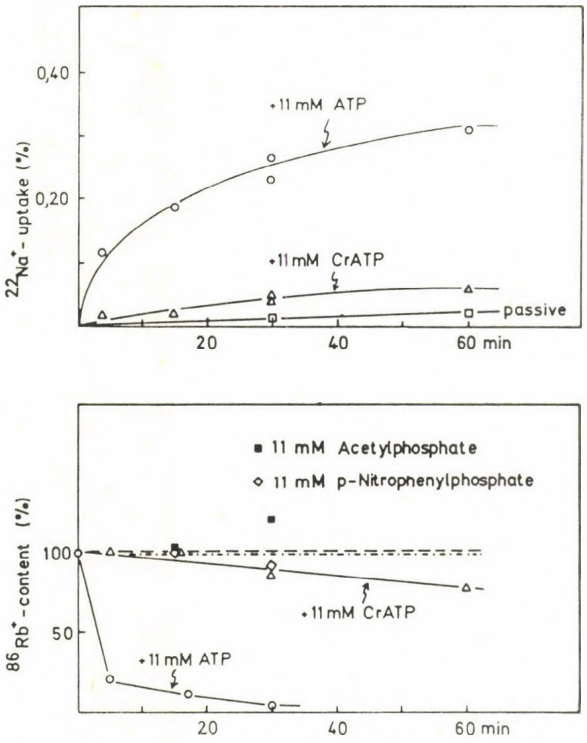


Fig. 4:
Evaluation of the substrate function of ATP, CrATP, acetylphosphate and p-nitrophenylphosphate to fuel the cation transport in liposomes containing purified $(Na^+ + K^+)$-ATPase from rabbit kidney. The transport was studied with liposomes containing 50 mM Na^+ + 50 mM Rb^+ in a transport medium containing the same composition described in (35).

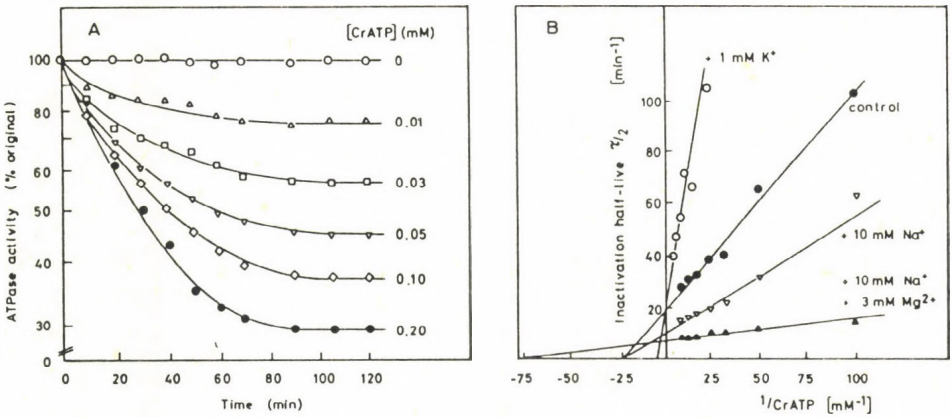


Fig. 5: Inactivation of $(Na^+ + K^+)$-ATPase from pig kidney by CrATP and study of the effects of Na^+, K^+ and Mg^{2+} on it (from refs. 36, 37). A) Time course of the inactivation at different concentrations of the α,β,γ-tridentate of CrATP. B) Secondary plot of the effects of Na^+, K^+ and Mg^{2+} on the rate of inactivation as a function of the CrATP concentration.

inactivation by CrATP (Fig. 5B) as it has to be expected for a substrate of $(Na^+ + K^+)$-ATPase. The half-maximal enhancement of the inactivation was seen at 6 mM Na^+. This may mean that a high affinity site for Na^+ - presumably the Na^+-transport site - may by its occupancy induce an alteration of the enzyme leading thus to a better inactivation of the enzyme. Although only small concentrations of Mg^{2+} are necessary to allow the Na^+-dependent phosphorylation in the overall-reaction of $(Na^+ + K^+)$-ATPase (17, 30), high concentration of Mg^{2+} further stimulates the CrATP inactivation of the enzyme (Fig. 5B). Half-maximal stimulation was seen at 1 mM Mg^{2+}. A possible function of this low affinity Mg^{2+} binding site leading to an enhancement of the inactivation will be shown later.

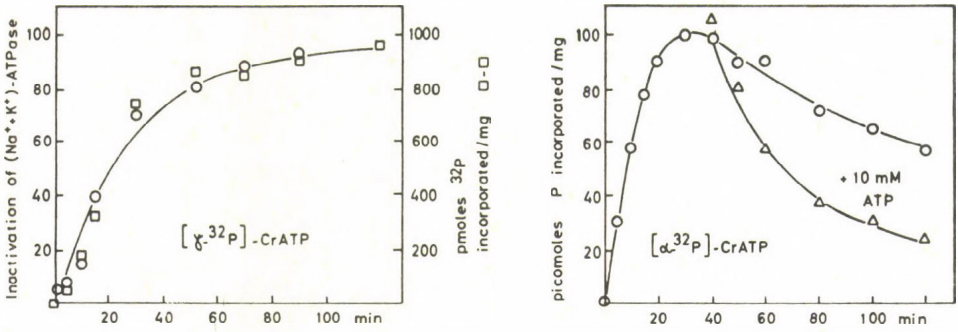


Fig. 6: Comparison of the incorporation of radioactivity from (α-^{32}P) and (γ-^{32}P)CrATP's into the protein of $(Na^+ + K^+)$-ATPase and demonstration of its inactivating effect. 0.1 mM CrATP was used (from ref. 36).

The stimulatory effect of Na^+ on the inactivation process by CrATP points out the possibility that $(Na^+ + K^+)$-ATPase might be phosphorylated during the inactivation. Indeed, as seen in Fig. 6A, the terminal phosphate from (γ-^{32}P)CrATP is transferred to the enzyme protein with a similar rate as the inactivation proceeds. Similar results were obtained with ^{51}CrATP demonstrating the formation of a chromo-phosphointermediate (36, 37). Consistent with the substrate function of CrATP for the Na^+-dependent proteinkinase reaction, the first step in the

overall reaction of $(Na^+ + K^+)$-ATPase (17, 34), we found only a transient acid stable incorporation of radioactivity from (α-^{32}P)CrATP (Fig. 6B) indicating the formation of a more stable CrATP enzyme complex prior to the phosphorylation and the release of (α-^{32}P)ADP. The finding that even the non-hydrolyzable 5'(β-γ imido)triphosphate derivative of CrATP inactivated $(Na^+ + K^+)$-ATPase (36, 37) is consistent with the above.

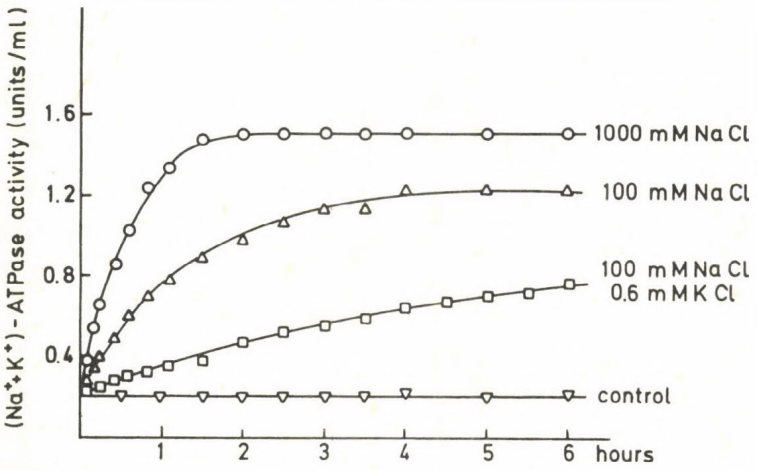


Fig. 7: Study of the effect of Na^+ and K^+ on the by CrATP inactivated $(Na^+ + K^+)$-ATPase (from ref. 40).

Since the inactivation of $(Na^+ + K^+)$-ATPase proceeds with its phosphorylation it is apparent that rate of dephosphorylation is even slower and that the inactivation of the enzyme has to be ascribed to the stability of the chromo-phosphointermediate. The hydrolysis of the magnesium-phosphointermediate formed from MgATP is enhanced by K^+ (17, 34). It is therefore plausible to look for the effects of univalent and divalent cations on the dephosphorylation and reactivation of the chromo-phosphoenzyme. Contrary to the expectations K^+ could not activate the hydrolysis of the chromo-phosphointermediate, but the Na^+ of high concentration could (Fig. 7). The half-maximal effects of Na^+ on the reactivation were seen at 40 mM and 126 mM, respectively, depending on the parameter studied. This concentration points out an extracellular attack of Na^+. K^+ ions counter-

acted the Na^+ effect with an apparent affinity of 70 µM (37, 40, 41).

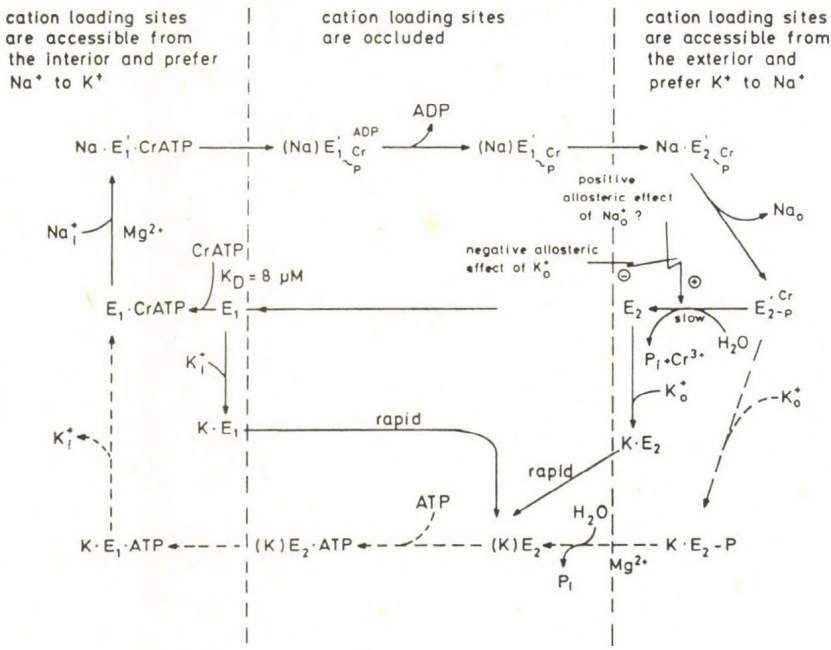


Fig. 8: Modification of the reaction scheme of Karlish et al. (44) showing relationships among ion movements, phosphoryl transfer and conformational changes proposed to occur in the presence of CrATP and its absence (solid lines) or in the presence of MgATP (dashed lines). MgATP is assumed to behave similarly to CrATP with the exception that Mg^{2+} is incorporated in the enzyme protein instead of Cr^{3+} and that the turnover is considerably faster.

Apparently CrATP allows only one half of the overall reaction of $(Na^+ + K^+)$-ATPase to proceed (Fig. 8). A possible explanation for the missing reactivity of the chromo-phosphointermediate as a substrate for the K^+-dependent phosphoprotein phosphatase reaction is the assumption, that Mg^{2+} has to be in the active site to convert the phosphoenzyme into the reactive, K^+-accepting form (27) (Fig. 8). Since this E_2-form is characterized by its capability to bind and occlude K^+ (42, 43), it is interesting to mention that the chromo-phosphoenzyme can not

occlude K^+ nor has it the capability to bind $^{86}Rb^+$ (a congener of K^+) at the high affinity site for K^+ (37). Since $(Na^+ + K^+)$-ATPase appears to proceed with CrATP only through one half of the reaction cycle, we looked for the possibility that CrATP may allow a Na^+-Na^+-exchange reaction, a partial reaction of the $Na^+ + K^+$-pump (17). Preliminary experiments with everted red blood cells indicate that this might be the case (40, 41). Since CrATP allows to occlude Ca^{2+} in the Ca^{2+}-ATPase of the sarcoplasmic reticulum in a stable form (33), we, furthermore, looked for the possibility that whether Na^+ is included in the $(Na^+ + K^+)$-ATPase in the presence of CrATP. Unfortunately, we have so far been unable to demonstrate this.

INDICATIONS FOR CATION-INDUCED ALTERATIONS OF THE ATP BINDING SITE

The studies on the MgATP complex analogue CrATP demonstrate that free Mg^{2+} in millimolar concentrations enhances the inactivation and phosphorylation of the enzyme (Fig. 5B). This enhancing effect of Mg^{2+} by its binding to a low affinity site may be brought about by an alteration of the affinity for the MgATP substrate, which is also seen in Fig. 5B. Since we found in independent equilibrium binding experiments, that Mg^{2+} in millimolar concentrations decreased the dissociation constant of the enzyme for the β,γ-methylene derivative of ATP from 20 µM to 2 µM (26), we were interested to see, whether these alterations in the ATP affinity can be ascribed to structural alterations of special parts of the ATP binding site. If the reactivities of the protein-reactive ATP analogues were sensitive to the cation environment, such an analysis would be possible.

Fig. 9 shows that neither types of azido-ATP photoinactivate $(Na^+ + K^+)$-ATPase in the absence of Mg^{2+} although both analogues are bound from the enzyme (15, 16). Such phenomenon can only be explained by the assumption that the binding of Mg^{2+} at a low affinity site alters the conformation of the enzyme making thereby an exposed reactant for the photoaffinity label which is otherwise not available. Indications for such a Mg^{2+}-induced

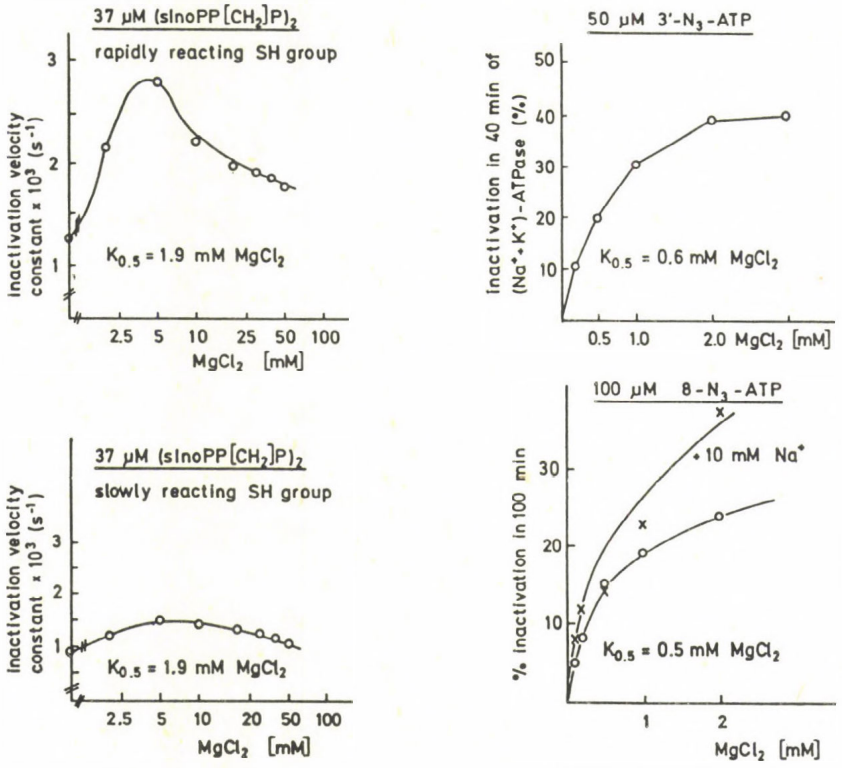


Fig. 9: Effect of Mg^{2+} on the modification of the ATP binding site by protein reactive ATP analogues (from refs. 15, 16, 29).

alteration are also available from the protective effect of Mg^{2+} against the trypsinolysis under controlled conditions (15). Mg^{2+} ions also enhance the inactivation of $(Na^+ + K^+)$-ATPase by $(sITP)_2$ (29, 40) (Fig. 9). This enhancing effect which does not affect the affinity for $(sITP)_2$ of the enzyme (29, 40) is more pronounced at the high affinity ATP binding site represented by the rapidly reacting sulfhydryl group. Similar but less pronounced effects were observed in the presence of Na^+ (15, 29) (Fig. 9). K^+ ions counteract the inactivation of $(Na^+ + K^+)$-ATPase with each ATP analogue. It is therefore plausible to assume that the ATP binding site of $(Na^+ + K^+)$-ATPase is tightened in the presence of Mg^{2+} and Na^+ but widened in the presence of K^+ (Fig. 10). The data on the protein-re-

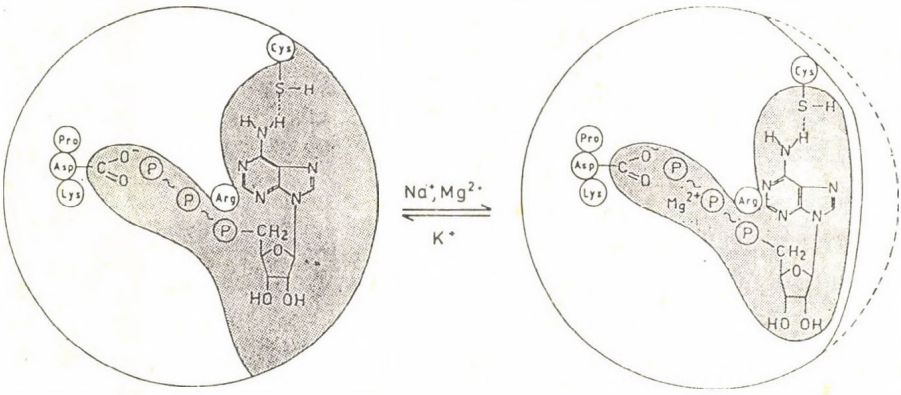


Fig. 10: Model on the effects of Na^+, Mg^{2+} and K^+ on the size of the ATP binding site. The amino acids participating in the recognition and hydrolysis of ATP are taken from refs. (19-21, 34).

active ATP analogues demonstrate, that the cation-induced structural alterations of the ATP binding site cannot be ascribed to a special part or a subsite of this binding site.

ACKNOWLEDGEMENTS

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DISCUSSION

MARTONOSI:

Is the phosphorylation rate of the Na-K-ATPase by Cr-ATP a rather slow process?

SCHONER:

The Ca^{2+}-ATPase is phosphorylated by Cr-ATP similarly like $(Na^{+}+K^{+})$-ATPase occluding thereby $^{45}Ca^{2+}$ in a ratio of 1 mole of Ca^{2+} ions occluded per mole of enzyme phosphorylated (Serpersu, et al., Eur. J. Biochem. 122:347, 1982).

SOMOGYI:

Dr. Vodnyánszky and myself could demonstrate several years ago that the p-nitrophenylphosphatase activity of $(Na^{+}+K^{+})$-ATPase can be stimulated by molar concentration of ATP even in the presence of low concentration of Na^{+} and K^{+} so long till unhydralyzed ATP is available in the measuring system. We could differentiate between two possibilities a.) the binding of ATP to the enzyme is needed for this stimulating effect and b.) the phosphoenzyme formation is necessary. My question is: among ATP analogues you synthetised is there any which can bind to the ATPase without its concomitant hydrolysis?

SCHONER:

Unfortunately I cannot name you any ATP analogues which can not be split and allows nevertheless the Na^{+}-dependent hydrolysis of p-nitrophenylphosphate via the partial reaction of the phosphatase. At least inactivation of $(Na^{+}+K^{+})$-ATPase by Cr-ATP by the phosphorylation also leads to an inactivation of the K^{+}-activated phosphatase. We did not look, however, for the possibility whether also the Na^{+}+ATP activated p-nitrophenylphosphatase, too, is altered in the same way.

Symposia Biologica Hungarica 26 (1984)

THE PROPERTIES OF MYOMETRIAL Ca^{2+}-TRANSPORT IN PREGNANT AND NON-PREGNANT RATS

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INTRODUCTION

Calmodulin is known to play an essential role in many intracellular Ca^{2+}-dependent processes including even calcium-transporting systems (Cheung, 1980). The calcium transport stimulating action by calmodulin was first described for erythrocyte plasma membrane Ca^{2+}-ATPase activity (Jarett and Penniston, 1977). Later it has been shown that calmodulin stimulates the active Ca^{2+}-transport both in myocardial sarcoplasmic reticulum (Katz and Remtulla, 1978) and plasma membrane (Caroni and Carafoli, 1981). The active Ca^{2+}-transport of gastric (Wuytack et al., 1981) or tracheal (Hogaboom and Fedan, 1980) smooth muscle microsomes is enhanced by calmodulin isolated from other tissues.

No direct data are available concerning the effect of calmodulin on Ca^{2+}-transport in myometrial smooth muscle, only an inhibition of Ca^{2+}+Mg^{2+} ATPase by a calmodulin antagonist compound trifluoperazine (TFP) was demonstrated in this tissue (Soloff and Sweet, 1982). The present examination deals with the calmodulin activated Ca^{2+}-transport of myometrial microsome fractions isolated from non-pregnant and pregnant rats. In addition a possible relationship between calmodulin and cyclic AMP dependent modulation of Ca^{2+}-transport in myometrial smooth muscle was studied.

METHODS

Female rats of CFY strain of the same age, but at different times of gestation were used. The first day of gestation can be estimated with one day error in the knowledge of the time of mating. Non-pregnant rats were treated with diethylstilbestrol for 3 days at a dose of 1 mg/kg b.w. per day in order to ensure identical hormonal influcences for the mymetrium of all animals. Uteri removed either from non-pregnant or pregnant (18 days) rats were homogenised in medium containing 0.25 M sucrose and 0.04 M histidine (pH 6.8). This was completed by 0.001 M EDTA to remove endogenous calmodulin when required. Myometrial microsomes were prepared according to the methods of Nishikori (1979). Microsomal Ca^{2+}-uptake was studied within two hours after the preparation using the methods of Wuytack (1980).

Membrane phosphorylation was estimated as described by Kranias (1980) except $2x10^{-4}$ M ATP were used.

RESULTS AND DISCUSSION

Previously we have reported, that the active Ca^{2+}-uptake measured with or without oxalate increased significantly with the time of gestation (Somogyi et al., 1983). The maximal Ca^{2+}-uptake was noted on the 18th day of pregnancy. According to the data in Table 1 the active Ca^{2+}-uptake of myometrial microsomes prepared either from non-pregnant or pregnant animals proved to be TFP sensitive.

Ca^{2+}-uptake determined in the presence of oxalate declined by 30 percent in the non-pregnant group whereas on the 18th day of pregnancy dropped by 60 percent. The exogenously added calmodulin did not essentially influence the active Ca^{2+}-uptake, this indicating that the studied preparations contained enough calmodulin to ensure maximal Ca^{2+}-uptake. Other Ca^{2+}-transport systems containing endogenous calmodulin also show a similar lack of sensitivity of exogenous calmodulin (Kanagasuntheran and Teo, 1982).

More direct evidence for the involvement of calmodulin in myometrial Ca^{2+}-transport was demonstrated on calmodulin depleted microsomes, which were prepared as the not-depleted ones except 1 mM EDTA was used in homogenising the medium (Table 2).

Table 1

Effect of trifluoperazine and exogenous calmodulin on active Ca^{2+}-uptake in calmodulin not-depleted microsome fractions of rat myometrium

Day of gestation	0		18	
Addition	ATP	ATP + oxalate	ATP	ATP + oxalate
no	5.3±0.3	17.9±1.3	8.3±0.5	28.9±1.7
50 μM TFP	4.0±0.3	12.3±0.7	4.6±0.2	11.6±1.3
10 μg/ml calmodulin	5.6±0.5	20.4±1.5	8.9±0.4	28.9±2.0

Average values of 4-8 experiments (±S.E.)
Ca^{2+}-uptake: nmoles $Ca^{2+} \cdot mg\ protein^{-1} \cdot 5\ min^{-1}$

Table 2

Effect of trifluoperazine and exogenous calmodulin on active Ca^{2+}-uptake in calmodulin-depleted microsome fractions of rat myometrium

Day of gestation	0		18	
Addition	ATP	ATP + oxalate	ATP	ATP + oxalate
no	6.5±0.5	13.7±0.9	5.2±0.6	19.1±0.3
50 μM TFP	6.2±0.4	13.1±0.9	8.6±0.5	18.2±1.0
10 μg/ml calmodulin	7.1±0.6	20.2±1.5	10.5±1.6	30.1±3.2

Average values of 4-8 experiments (±S.E.)
Ca^{2+}-uptake nmoles $Ca^{2+} \cdot mg\ protein^{-1} \cdot 5\ min^{-1}$

The Ca^{2+}-uptake of calmodulin depleted myometrial microsomes was considerably lower than the Ca^{2+}-uptake of not-depleted preparations. 50 μM of TFP remained without effect, on the other hand calmodulin applied in supramaximal concentration to these preparations enhanced Ca^{2+}-uptake. All these data appear to indicate that endogenous calmodulin can be removed from myometrial microsomes after a relatively mild treatment, but the original Ca^{2+}-uptake rate can be restored by the addition of exogenous calmodulin.

In calmodulin not-depleted preparation 10 μM cyclic AMP did not essentially influence the Ca^{2+}-uptake. However, when trifluoperazine was applied simultaneously with cyclic AMP, no inhibition of Ca^{2+}-uptake was observed. This effect might be explained by the action of the endogenous cyclic AMP dependent protein kinase. This idea is supported by our very recent observation: in the presence of an cyclic AMP dependent protein kinase inhibitor the protective effect of cyclic AMP mostly could not prevail at all. Cyclic AMP dependent membrane phosphorylation relating to the regulation of myometrial Ca^{2+}-transport is suggested by another report, too (Nishikori and Maeno, 1979).

In calmodulin not-depleted myometrial microsomes 50 pmole ^{32}P/mg protein/20 sec was incorporated in the presence of 10 μM Ca^{2+}. This phosphorylation was further increased by the addition of cyclic AMP. In contrast to the Ca^{2+}-uptake measurements the exogenous calmodulin significantly enhanced the ^{32}P incorporation in these preparations.

It can be concluded that the Ca^{2+}-transport in myometrial smooth muscle is regulated by calmodulin. It also can be suggested, that calmodulin as well as cyclic AMP dependent membrane phosphorylation might be related to the regulation of myometrial Ca^{2+}-transport. However, the exact explanation of the relationship between the calmodulin and cyclic AMP-pathway in this process needs further detailed investigations.

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DISCUSSION

SCHATZMANN

What were the conditions (Ca^{2+}-concentration exposure time, temperature) in the phosphorylation experiments?

VÉR:

The phosphorylation experiments were done at 30°C in the presence of 10 μM free Ca^{2+} concentration, at pH 7.2 during 30 sec.

IKEMOTO:

I think this is one of the first reports about the smooth muscle calcium-pump system. Could you give us some of the basic information; e.g., protein composition and time course of Ca^{2+} uptake, etc.?

VÉR:

The applied microsome fraction contains both sarcolemmal and internal membrane (SR) proteins. More than 40 bands could be observed in SDS gelelectrophoresis. The exact characterization of compositions are in progress. The active Ca-uptake was linear at least for 10 min, the incubation was carried out at 37°C.

FONYÓ:

Two technical questions:
How can you deplete your system of endogenous calmodulin, and
How can you know how efficient your depletion procedure was?

VÉR:

For endogenous calmodulin depletion we used 1 mM EDTA in homogenasing medium.
The used depletion procedure seems to be effective enough, since TFP cannot decrease the active Ca^{2+}-uptakes in calmodulin depleted preparations any longer.

MARTONOSI:

The activation of the calcium transport by calmodulin indicates the participation of surface membrane elements while the oxalate activation of Ca^{2+} transport is usually attributed to elements derived from sarcoplasmic reticulum. Could you indicate the relative contribution of these two Ca^{2+} transport systems to your microsome fraction?
Is the phosphoprotein formed under your conditions hydroxylamine sensitive or insensitive?

VÉR:

The ratio of sarcolemmal and internal membrane (SR) system in microsomal preparation can be approximately estimated after comparing the specific activity of plasma membrane marker Na^+/K^+-ATPase in microsomal and purified plasma membrane preparations. In the case of myometrium the problem was that no Na^+/K^+-ATPase activity could be detected, without a sufficient detergent treatment. However, detergent treatment. However, detergent treatment caused an irreversible loss in Na^+/K^+-ATPase activity in the microsome and plasma membrane (personal communication by Ágnes Fari).
Most of the detected phosphoprotein was hydroxylamine insensitive, in our conditions.

DUX:

Have you observed any effect of pregnancy on the phosphoprotein formation?

VÉR:

The experiments are in progress.

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CONTROL BY MAGNESIUM IONS OF THE MONOVALENT CATION/PROTON EXCHANGE IN MITOCHONDRIA

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Potassium ion is the main cation of mitochondria. For example, its concentration in the matrix of heart mitochondria amounts to about 140 mM, whereas that of sodium ion is 30 mM or less (1). These are the concentrations similar to those of both cations in the cell cytoplasm (2). In spite of this, the mitochondrial inner membrane is not freely permeable to monovalent alkali metal cations. This is understandable, since a high permeability would result in an enormous accumulation of these cations in the matrix compartment due to the electrochemical potential (negative inside) which exists at the inner mitochondrial membrane under energized conditions. Nevertheless, a low electrophoretic influx of K^+ does occur in freshly isolated essentially intact mitochondria and it is, most likely, characteristic of mitochondria in situ. To maintain a constant content of mitochondrial potassium, this influx must be compensated by an extrusion of K^+ (3). This is operated by a K^+/H^+ exchange mechanism (Fig. 1).

Potassium transport across the inner mitochondrial membrane has extensively been studied during last two decades (reviews 1, 4-6). The aim of the present article is to recollect information on the regulation of the monovalent cation/H^+ (in particular K^+/H^+) exchange, with a special attention to the role of magnesium in controlling this process.

Extrusion of monovalent cations from mitochondria by energization was observed already in 1967, by Azzi and Azzone (7) and the operation of the electroneutral monovalent cati-

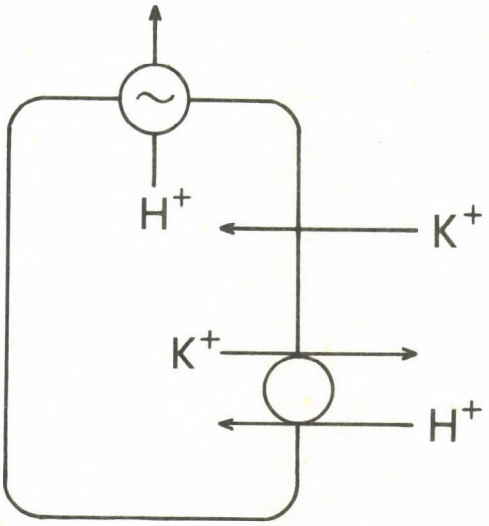

Fig. 1:
Pathways of K^+ transport in mitochondria: the electrophoretic uptake and the electroneutral K^+/H^+ exchange. The energy-dependent (respiration- or ATP-driven) proton extrusion is indicated by ~.

on/proton exchange was explicitly proposed somewhat later (4, 8-11). Douglas and Cockrell (11) studied the specificity of the exchange towards different monovalent metal cations and found it to vary upon experimental conditions. Increased permeability of mitochondria to monovalent cations produced by chelating agents and its reversal by Mg^{2+} have also been long known (12-16). Wehrle et al. (17) found that a concerted effect of EDTA and the divalent metal ionophore A23187 (first described by Reed and Lardy, 18) greatly enhanced the permeability of beef heart mitochondria to monovalent metal cations. They observed that depletion of internal and membrane-bound magnesium increased the respiration-dependent uptake of these cations in the presence of acetate anion. It was also observed at the same laboratory (19) that Mg^{2+} competitively inhibited this uptake of K^+.

Our studies on rat liver mitochondria clearly showed that the electroneutral K^+/H^+ exchange was activated by magnesium depletion (20). Mitochondria suspended in isotonic KNO_3 swelled rapidly when the medium was supplemented with the ionophore A23187, EDTA and a protonphore, e.g. carbonyl cyanide-m-chlorophenylhydrazone, and the swelling was inhibited by adding Mg^{2+} (Fig. 2). In this experiment NO_3^- entered the internal mitochondrial compartment down the concentration gradient as a permeant anion (21), producing a transmembrane potential, neg-

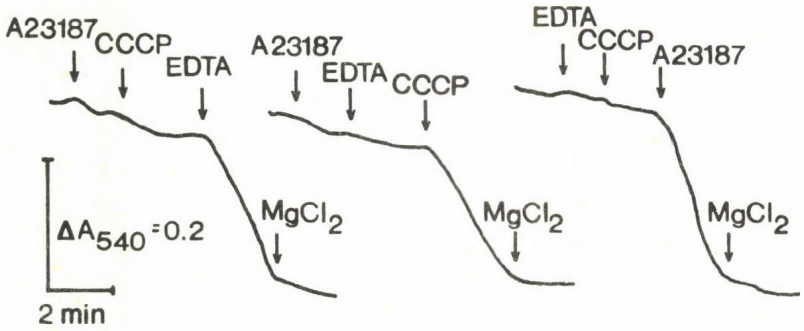

Fig. 2: Swelling of mitochondria in 135 mM KNO_3. Additions: ionophore A23187, 1 µM; carbonyl cyanide-m-chlorophenylhydrazone (CCCP), 2 µM; EDTA, 1.3 mM; $MgCl_2$, 10 mM. From (20).

ative inside. This was followed by the entry of K^+. However, since swelling did not proceed appreciably unless the protonophore was added (middle trace in Fig. 2), we concluded that a K^+/H^+ exchange rather than the electrophoretic influx of K^+ appeared as the result of magnesium depletion. The postulated mechanism is illustrated in Fig. 3.

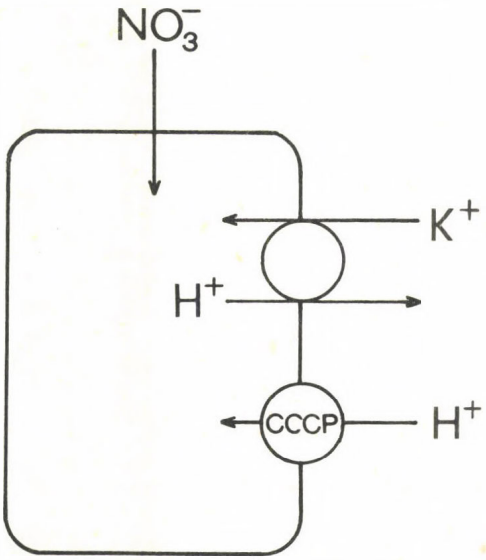

Fig. 3:
Scheme for the mechanism of mitochondrial swelling in isotonic KNO_3 produced by magnesium depletion. From (20), modified.

The role of intramitochondrial Mg^{2+} or another divalent metal cation in controlling the K^+/H^+ exchange was fully confirmed by Garlid and coworkers (22-24). By selectively depleting mitochondria of either Mg^{2+} or Ca^{2+} these authors have also been able to show that magnesium and not calcium ions play the role (25).

It has long been known that an increased permeability to K^+ and activation of the monovalent cation/H^+ exchange in mitochondria are also produced by mercurials and other thiol group reagents (3, 26-30). Data from our laboratory (31) suggest that this effect may also be due to the depletion of mitochondrial magnesium. Firstly, we demonstrated that p-hydroxymercuribenzoate increased the permeability of mitochondrial membranes to Mg^{2+} and produced a release of membrane-bound magnesium. Secondly, we found that mitochondrial swelling in isotonic KNO_3, produced by mercurials plus a protonophore, was prevented by Mg^{2+} (Fig. 4). Interestingly enough, the amphophilic cation cetyltrimethyl-ammonium also protected against swelling (this observation will be commented later on). It has to be mentioned, however, that Diwan et al. (32) do not agree with this interpretation and propose that the swelling action of mercurials can be explained by the inhibition of pH shifts associated with exchange of endogenous phosphate.

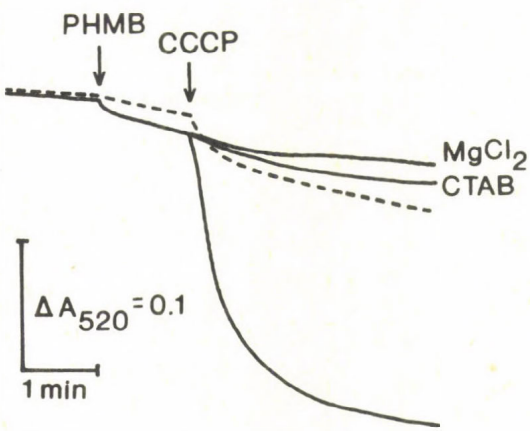

Fig. 4:

Swelling of mitochondria in isotonic KNO_3 produced by mercurials. The medium contained 130 mM KNO_3, 10^3 mM Tris-HCl (pH 7.4) and 3.2 mM $MgCl_2$ or 5 µM (30 nmol/mg protein) cetyltrimethyl-ammonium bromide (CTAB) where indicated. Additions: p-hydroxymercuribenzoate (PHMB), 2 µM (12.5 nmol/mg protein); carbonyl cyanide-m-chlorophenylhydrazone (CCCP), 1 µM. Dashed line represents the control to which no PHMB was added. From (31).

In view of what has been said so far the two following questions appear: first, what is the mechanism of the K^+/H^+ exchange and what is the nature of the relevant exchanger (if any) like; second, what is the actual molecular mechanism by which Mg^{2+} inhibits the exchange.

Concerning the first question, Blondin and colleagues (30, 33) have claimed to isolate a natural ionophore of polypeptide nature from beef heart mitochondria. However, more rigorous studies seem not to support this finding (cf. 5). On the other hand, I have previously shown that long chain non-esterified fatty acids and their thioesters with CoA increase the permeability of the mitochondrial membrane to monovalent metal cations (34). Assuming that undissociated fatty acids and their potassium salts can undergo a rapid transbilayer movement in the inner mitochondrial membrane, one may think that they can function as "natural" ionophores. Moreover, it has also been shown (34) that the increased permeability to K^+ induced by fatty acids is prevented by Mg^{2+}. This may be due to the formation of more stable and less mobile magnesium salts. This way of speculation can be extended over any acidic components of mitochondrial membranes, in particular phospholipids, if their potassium salts are stable enough to withstand the transbilayer passage. If so, it may not be necessary to assume the existence of a particular K^+/H^+ exchanger. The latter view is substantiated by the observation that a simple stretching of the membrane, i.e. loosening of its structure, e.g. by hypotonic swelling, increases the rate of the monovalent cation/H^+ exchange (35-37). However, the swelling is associated with the loss of intramitochondrial magnesium and therefore it might as well produce unmasking of the exchanger due to Mg^{2+} depletion (38). It is also interesting to note that conditions which favour the K^+/H^+ exchange, e.g. Mg^{2+} depletion, thiol group reagents and fatty acids, increase the respiration-dependent electrophoretic potassium uptake (e.g. 17, 19, 34) and, most likely, the proton permeability (29, 39). On this basis Azzone et al. (35, 36) proposed to regard the monovalent cation/H^+ exchange as a short-range coupling of

electric monovalent cation and proton fluxes. This view can be supported by a recent observation that the K^+/H^+ exchange is controlled by the transmembrane electrochemical potential rather than by the pH gradient (40).

Concerning the mechanism of the controlling effect of Mg^{2+} several possibilities can be considered as well. Firstly, Mg^{2+} may complex the putative exchanger, as already discussed, for free fatty acids (see also 34). Secondly, by forming complexes with anionic groups of membrane proteins and phospholipids, Mg^{2+} stabilizes the membrane in the sense of limiting the mobility of membrane components. This possibility was suggested by Ligeti and Fonyó (41) who observed that the valinomycin-induced K^+ uptake by mitochondria was competitively inhibited by Mg^{2+}. In fact, Ligeti and Horváth (42), using a spin-labeled membrane probe, recorded structural alterations (but not a change of membrane fluidity sensu stricto) of the inner mitochondrial membrane induced by Mg^{2+}.

Finally, the effect of Mg^{2+} may be ascribed to changing the surface potential of the mitochondrial membrane. It can be calculated (cf. 43) that the negative charge of a biological membrane of 0.05 C/m^2 produces an almost tenfold increase of the local concentration of monovalent cations in the immediate vicinity of the membrane. Consequently, variations of the surface potential should result in changes of this local concentration and thus affect the flux through the monovalent cation/H^+ exchanger. It has already been shown (44) that magnesium ions partly neutralize the negative surface charge of mitochondrial membranes and therefore this mechanism of regulating the K^+/H^+ exchange seems plausible. This explanation is also supported by the observation (Fig. 4) that the exchange can be inhibited not only by Mg^{2+} but also by a low concentration of the organic cation cetyltrimethyl-ammonium which exhibits a high affinity to biological membranes and can neutralize their surface potential (44). In a similar way the potentiating effect of fatty acids and acyl-CoA esters on the monovalent cation/H^+ exchange (34) might be explained by the fact that they increase the negative surface potential of

biological membranes (44), thus increasing the local concentration of the respective cation at the membrane.

Although in all studies on the role of magnesium in controlling monovalent cation fluxes in mitochondria the concentration of either external or intramitochondrial Mg^{2+} has been changed experimentally, it is highly likely that magnesium associated with the membrane rather than free Mg^{2+} is essential. In connection with this it is worthy to stress that in rat liver mitochondria 5 % of mitochondrial magnesium is bound to the inner membrane, whereas 50 % is released with components of the intermembrane compartment (45). In the latter fraction magnesium is bound to high molecular weight compounds, presumably proteins (46), and there is a good reason to suppose that in the intact mitochondrion this magnesium is associated with the inner membrane as well. Thus, membrane-bound magnesium seems to be an important factor in controlling the permeability of mitochondria to monovalent cations.

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DISCUSSION

SZAMEL:

Biological membranes contain very small amounts of fatty acids, can you tell the exact amount of free fatty acids in the inner membrane of mitochondria; can the amount of free fatty acids be correlated with the amount of potassium uptake?

Can the effect of free fatty acids on potassium transport be explained by their known detergent properties?

How do you explain the fact, that palmiroyl coenzyme A proved to be more effective in enhancing potassium uptake than free palmitic acid?

WOJTCZAK:

Freshly isolated rat liver mitochondria contain about 10 nmol non-esterified fatty acid per mg protein. It is, however, difficult to say whether all of this fatty acid is related to mitochondria in situ or some of it may have been formed during the isolation procedure.

In the presence of acetate (permeant weak acid) we observed an energy-dependent accumulation of K^+ in mitochondria induced by fatty acids. This cannot be ascribed to the detergent effect since detergents would produce a release, and not an accumulation, of potassium ions.

If the effect of fatty acids is due to the increase in the negative surface charge (and thus making the local concentration of K^+ higher), then acyl-CoA, being more negative than free fatty acids, should have a more potent effect.

SANADI:

There may be explanations of the stimulatory effects of mercurials on K^+ transport other than the so-called general permeability effect. Ca^{2+} produces a similar stimulations in the presence of an excess of 2-mercaptoethanol which makes Ca^{2+} more selective for dithiols. This effect

is not altered by ruthenium red which is known to inhibit Ca^{2+}-uptake into mitochondria. The Ca^{2+} bound tightly to mitochondria under these conditions is approximately 5 to 6-times the amount of cytochrome a, which makes the stimulatory effect relatively specific.
The question is, how does the A23187, FccP and EDTA induced swelling rate compare with the mercurial induced swelling rate?

WOJTCZAK:

Mitochondrial swelling induced by mercurials was of approximately the same rate as that produced by A23187 plus CCCP plus EDTA.

KEPES:

An interesting possibility could be contemplated in connection with the K^+ permeability after removal of Mg^{2+} namely that a potential barrier right in the case of the hydrophobic layer. In effect the electrical field created by a fixed charge propagates through the lipidic dielectric by the polarized water molecules. The moving charge of K^+ can be exposed to this electrical field even if it is bound to an intrinsic membrane protein.
An example from the field of bacterial transport can illustrate this hypothesis. Melibiose-sodium symport in E.coli is greatly stimulated by tiny amounts of TPB (tetraphenylboron, a lipophilic anion) while it is inhibited by tiny concentration of TPP^+ (tetraphenylphosphonium).

WOJTCZAK:

This is an interesting speculation, but we are rather inclined to the idea that magnesium is associated with the surface of the mitochondrial membrane and not with its lipidic hydrophobic core.

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A POSSIBLE ROLE OF CELL MEMBRANE IN REGULATION OF CELL DIVISION AND MALIGNANT TRANSFORMATION

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THE MODEL

It was recently proposed by one of us (Bhargava, 1974, 1975, 1977) that a resting cell is a resting cell because it is unable to take up essential nutrients at rates obtained in - and, apparently, necessary for - dividing cells. It was pointed out that all cells in which a true resting state is obtained are, without exception, auxotrophic for a number of carbon-containing nutrients. It was proposed that, in resting cells, only one of the two postulated functionally different transport sites for essential nutrients is open. When this site (Site A) operates at its maximal velocity, the uptake is just sufficient to take care of the requirements of the resting state. The second site (Site B) was proposed to be closed in resting cells through the interaction of a transport-inhibitory protein (made inside in the cell for translocation outside, and called $\underline{I}$) with its receptor on the cell membrane. The sites through which the transport machinery operates, irrespective of the mechanism of uptake, were suggested to be spatially linked to the site for the receptor for $\underline{I}$. Thus, $\underline{I}^+$ phenotype would mean that Sites B are closed and the cell would be a resting cell, and $\underline{I}^-$ phenotype will imply that Sites B are open and, therefore, the cell can go through the division cycle.

It was further suggested that mitogens act by making the cell $\underline{I}^-$, for example, by binding to a site (also spatially linked to the receptor site for $\underline{I}$) on the cell membrane in such a way that, following an allosteric transition, $\underline{I}$ can no longer bind to its own receptors. Malignancy, in terms of the model, would be the consequence of a situation where cells are not only phenotypically $\underline{I}^-$, but also no longer possess the ability to revert back to the $\underline{I}^+$ state. Such a situation could arise as a result of, for example, (a) mutation in the gene for $\underline{I}$ or for its receptor; (b) introduction of new information in the cell, say, through an oncogenic virus, which leads to the synthesis of a protein that gets associated with the cell membrane in such a way that $\underline{I}$ can no longer bind to its own receptor; or (c) mutation in the regulatory or the structural part of a host gene (e.g., the cellular counterpart of a viral oncogene) which codes - either in its normal or in its mutated form - for a product which migrates to the cell membrane and performs the same function as suggested above for a mitogen.

The $\underline{I}$ protein was suggested to be tissue-specific and possess, generally speaking, a low affinity for its receptor so that, normally, high concentrations of $\underline{I}$ would be required for it to occupy all of its receptors on the

cell surface and thus block transport through Sites B, thereby leading to a resting state.

The above-mentioned model, schematically described in Fig. 1, as far as we can see, explains the various states (the embryonic, the adult resting, the adult dividing, and the malignant) of cells in higher organisms, and the transition from one state to another (for example, embryonic to adult resting; adult resting to adult dividing as happens in liver regeneration or wound healing; adult dividing to adult resting as occurs, for example, in density-dependent inhibition of growth in tissue culture; normal to malignant, as can be achieved through certain physical and chemical agents or through oncogenic viruses; and malignant to normal, as appears to be the case in certain instances of somatic cell hybridisation between normal and malignant cells) that one encounters (Bhargava, 1977, 1983 a,b). The model, however, does not provide an explanation for tumours such as crown galls in plants, and leukaemias and teratomas in animals which almost certainly are a consequence of a block in differentiation and are, therefore, different from other tumours (such as hepatomas) which result from a permanent disturbance in the normal mechanism of regulation of cell division.

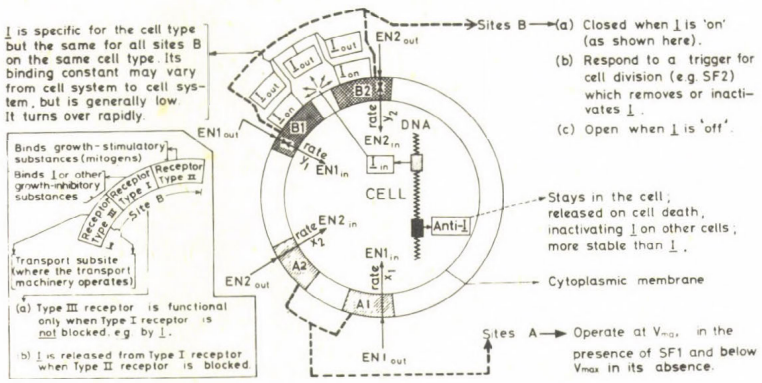

Fig. 1. A model for the regulation of cell division through control of uptake of essential nutrients. EN1 and EN2, essential nutrients; Sites A1 and A2, membrane sites for the uptake of various ENs in resting cells (these sites may also be open in dividing cells); Sites B1 and B2, membrane sites for the uptake of ENs in dividing cells, closed in resting cells; rate x, the maximal rate (V_{max}) of uptake of an EN through a Site A; rate y, the maximal rate of uptake of an EN through a Site B, probably between 5x and 10x; SF1, a serum factor necessary for transport of an EN through Site A at the maximal rate; I, an inhibitor of transport through Sites B, which comes out of the cell and acts from outside. Anti-I, an intracellular factor, functionally antagonistic to I and normally incapable of coming out of the cell; SF2, a serum factor functionally antagonistic to I. The main figure shows a resting cell. Sites B (inset left) are postulated to consist of three subsites, named Type I, Type II and Type III receptors; morphological overlap of these receptors is not ruled out (Bhargava, 1977).

The main prediction of the model is the existence of a transport-inhibitory protein, I, on the surface of resting cells, which protein would satisfy the following criteria: (a) it would bring down the high rate of uptake of essential nutrients such as the essential amino acids, obtained in dividing cells, to the low level obtained in homologous resting cells; (b) it would have no effect on the low rates of uptake obtained in resting cells; (c) as a rule, it would have a high rate of turnover; (d) it would be able to exercise a trans effect on those malignant cells in which the malignancy was caused by mutation in the gene for the I protein; (e) it would inhibit division in the above type of malignant cells as well as in homologous normal cells; (f) it would, therefore, also inhibit DNA synthesis in these cells.

THE EXPERIMENTAL APPROACH

We felt that a good system for the isolation of the I protein would be adult liver as, in this tissue, almost all the cells are resting even though they retain the capacity to go into the division cycle, as they do following partial hepatectomy. The parenchymal cells of adult liver would, therefore, be I^+. Dispersion of the liver tissue to a single cell suspension would be expected to lead to the dissociation of I from the surface of the parenchymal cells, if the affinity of I for its own receptors is not very high, as has been postulated in the model. The resulting primary liver parenchymal cell suspensions should then show the high rates of uptake, e.g. of essential amino acids, characteristic of dividing cells. This has already been demonstrated (Bhargava & Bhargava, 1962; Bhargava et al., 1975). The cells would, however, retain the ability to make I and, therefore, in dense cell suspensions could make sufficient I to reduce the rate of transport. In other words, if the above model is valid, the rates of uptake of amino acids should be found to depend on the concentration of the cells in a freshly prepared liver parenchymal cell suspension. This was, in fact, observed by us much before the model was constructed (Bhargava & Bhargava, 1962; Bhargava et al., 1975). We had called the phenomenon, "the cell concentration effect".

On dispersion of rat liver to a primary cell suspension and centrifugation of the suspension to sediment the cells, the I protein should appear in the supernatant. Following the above logic, we have attempted over the last ten years, to isolate a protein from the above-mentioned supernatant (which we have called the intercellular material fraction, or ICM, for convenience), which protein would satisfy the criteria already stated for I. In the last two years, we have succeeded in purifying to homogeneity such a protein from the ICM of rat liver. During its purification, it was assayed by its ability to (a) bring down the high-level uptake of certain amino acids obtained in cells from normal rat liver incubated at low cell concentrations, to the low-level uptake obtained in the high-concentration cells; (b) inhibit the uptake of essential nutrients such as amino acids, as well as, consequentially, DNA synthesis, in liver cell suspensions obtained from partially hepatectomised animals; (c) bring down the high-level uptake of essential amino acids obtained in a liver tumour (we have used the Zajdela ascitic hepatoma (ZAH), a benzpyrene-induced tumour) to the low-level uptake obtained in resting liver cells; (d) inhibit DNA synthesis, as a consequence of inhibition of the uptake of amino acids (as predicted in the model), in the ZAH. The rationale for the above assays for I has already been described in detail elsewhere (Bhargava et al., 1979).

We have also obtained evidence suggesting that, in the ZAH, the above-mentioned normal rat-liver I protein may be structurally altered. We now describe below the preparation and properties of the I and the I-like protein from normal rat liver and the ZAH, respectively.

THE I PROTEIN FROM NORMAL RAT LIVER

Two methods for the preparation of I from normal liver and from the ZAH have now been standardised in our laboratory (Figs. 2-4). The ICM was obtained from 6-8 month-old rats following dispersion of rat liver essentially as described by Jacob & Bhargava (1962), in Earle's base buffered with HEPES (0.04 M, 1-3 w/v) to pH 7.4 (this medium was used throughout). The cell suspension was centrifuged at 1000 x g at room temperature, and the supernatant centrifuged first at 20000 x g for 15 min and then at 105000 x g for 2 h; the resulting supernatant (the ICM) was chromatographed on a DEAE-Sephadex A-50 column (4.5 x 30 cm) equilibrated with the Earle's HEPES buffer. The adsorbed protein was eluted with 2M NaCl in the same buffer (Fig. 2A); peak II, which contained the I protein, was processed differently in the two methods.

In the first method (method 1), the above peak II was, after dialysis and lyophilisation, passed through a Biogel P-200 column (2.8 x 120 cm) prepared in Earle's-HEPES buffer. Of the several peaks generally obtained (Fig. 2B), peaks II and III were found to be biologically active as assayed using one or more of the above-mentioned assay systems. Peak II consisted of pure, homogeneous I protein. It gave a single band on non-SDS-PAGE (Fig. 5B; compare with the gel run of the DEAE peak II in Fig. 5A) using Coomassie Blue for staining, two very close bands on SDS-PAGE using silver for staining (suggesting that it may consist of two polypeptide chains of approx. 50,000 mol. wt; Fig. 6A), a single peak on high performance liquid chromatography (HPLC) using a hydrophobic C-18 column, and a single precipitin band with antisera raised against it in several rabbits.

In the second method (method 2), the material eluted in peak II from the DEAE-Sephadex A-50 column, was chromatographed (Fig. 3A) on an immunoadsorbant column prepared from antisera raised either against I purified by the first method or against one of the I fractions (Fig. 4A, fraction 2) obtained following fractionation of the material eluted from the above immunoadsorbant column (Fig. 3, peak I) on HPLC; the two antisera are called AS1 and AS2, respectively. The immunoadsorbant column was prepared in the usual way by CNBr-coupling of the protein precipitated from the antiserum made in rabbit with 40% saturation of ammonium sulphate, with Sepharose. When the DEAE-adsorbed protein peak II (Fig. 2A) was run on the antibody-Sepharose column (2.5 x 20 cm), and the protein adsorbed on this column eluted with 0.2M glycine-HCl buffer, two protein peaks were obtained, one of which eluted at pH 3.0 and the other at pH 2.2. Both the protein peaks had biological activity. When these peaks were run on a Waters I-250 gel filtration HPLC column (Fig. 4A,B), 3-5 peaks were generally obtained.

The following evidence, partially summarised in Table 1 and illustrated in Figs. 4-6, shows that the various HPLC fractions of the two protein peaks (peaks I and II) obtained from the immunoadsorbant column, these peaks themselves, and peak II from the Biogel column, all represented mixtures containing different proportions of various oligomers of the protein, and that some of the oligomers may have antigenic determinants not shared by the monomer:

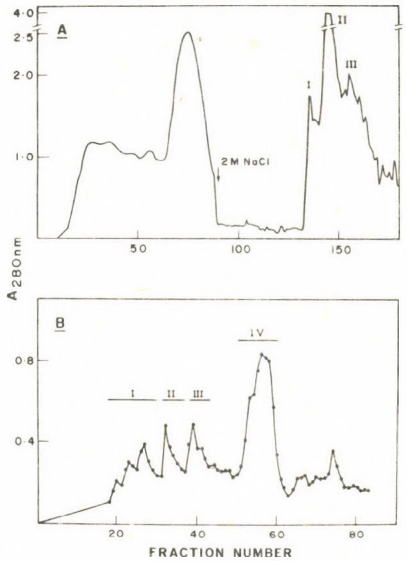

Fig. 2. Purification of the I protein from rat liver. ICM (after centrifugation) was chromatographed on a DEAE-Sephadex A-50 column (A). Peaks I and II showed activity. Peak II was dialysed, lyophilised and chromatographed on a Biogel P-200 column (B); peaks II, III and IV were active. The Biogel column peak II appeared homogeneous on PAGE and HPLC.

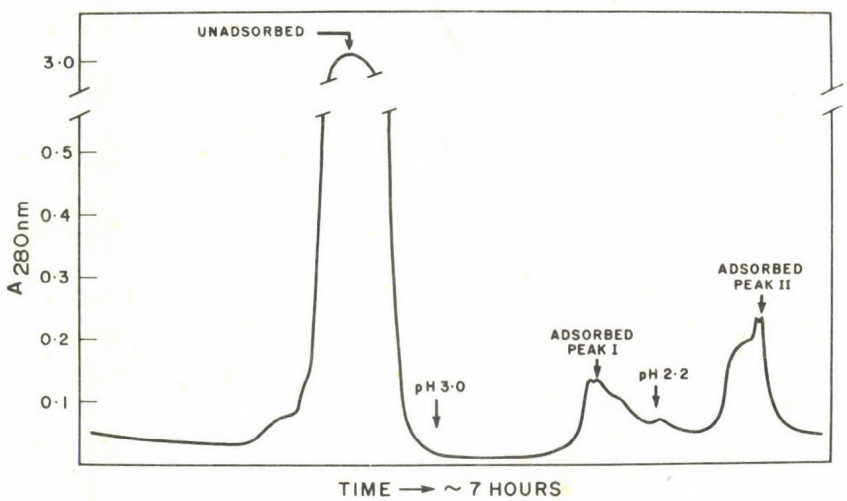

Fig. 3. Chromatography on an immunoadsorbant column, of the active I-containing fraction (peak II) from the DEAE-Sephadex A-50 column. For details of the A-50 column, see Fig. 2A. Details of the immunoadsorbant column are given in the text.

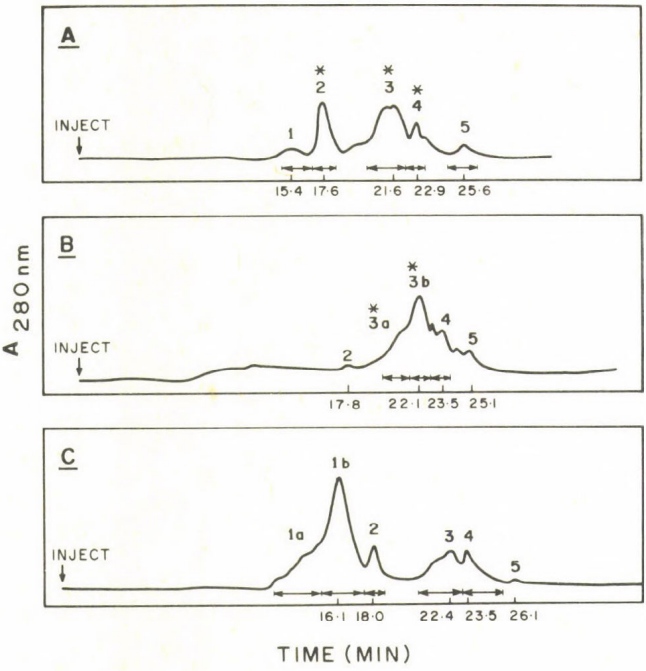

Fig. 4. HPLC runs of the two peaks of I-containing fractions eluted from the immunoadsorbant column. The HPLC was done on two Waters I-250 molecular sieve columns placed in tandem. A, HPLC run of immunoadsorbed peak I (Fig. 3) derived from ICM of normal rat liver. B, HPLC run of immunoadsorbed peak II (Fig. 3) derived from ICM of normal rat liver. C, HPLC run of immunoadsorbed peak I (obtained following the procedure of Figs. 2A and 3) derived from the ascitic fluid of the ZAH. Asterisks indicate the active peaks. The correspondence of the peaks in the three runs shown here was established by PAGE. The small differences (± 0.5 min) in elution times are due to slight variations in the conditions used for HPLC.

(a) All the above fractions gave the same two very close (which merged at times into one) bands on SDS gels using silver for staining.

(b) In non-SDS gel runs, using silver stain, upto four bands were obtained in the various fractions. These bands fitted into an oligomeric pattern on the basis of estimation of their apparent mol. wt. from their position on the gels; these mol. wts. corresponded to the monomeric, dimeric, trimeric and tetrameric forms of the protein. It was also clear that the protein consisted predominantly of the monomer; the di, tri and tetramer were not detected by the less-sensitive Coomassie Blue stain. As the silver stain, on account of its high sensitivity, could detect even minor constituents, and as (often) the gels were deliberately overloaded to detect such constituents, oligomers which could not generally be detected using Coomassie Blue were detected when silver was used for staining.

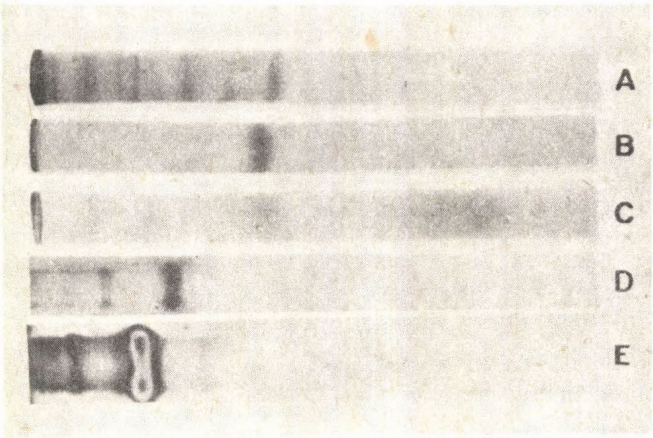

Fig. 5. Non-SDS-PAGE of the I protein from normal liver and the ZAH. A, I protein-containing fraction from normal liver (DEAE A-50 adsorbed peak II, Fig. 2A); B, I protein from normal liver (Biogel P-200 peak II, Fig. 2B); C, I protein from the ZAH (Biogel P-200 peak II); D, I protein from normal liver (fraction 3 of the HPLC run of the antibody-Sepharose column peak I; Fig. 4A); E, I protein from the ZAH (fraction 2 of the HPLC run of the antibody-Sepharose column peak I; Fig. 4C); A, B, C, Coomassie Blue staining; D, E, silver staining. The five gel runs shown are from separate experiments.

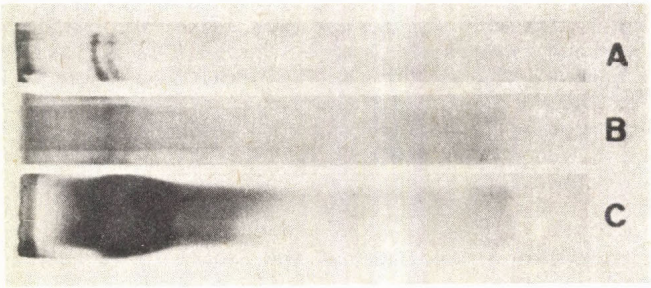

Fig. 6. SDS-PAGE of the I protein from normal rat liver and the ZAH. A, I protein from normal liver (Biogel P-200 peak II; Fig. 2); B, I protein from normal liver (fraction 3 of the HPLC run of the antibody-Sepharose column peak I; Fig. 4A); C, I protein from the ZAH (fraction 2 of the HPLC run of the antibody-Sepharose column peak I; Fig. 5C).

(c) The HPLC fractions that eluted earlier on the gel filtration column used for the HPLC runs, contained a higher proportion of the higher molecular weight oligomers than did the fractions that eluted later.

It would obviously be interesting to study the conditions which lead to oligomerisation of the I protein. All of the above fractions that were tested (the I protein obtained by method 1, the two fractions obtained from the immunoadsorbant column, and several of the HPLC fractions) were found to be biologically active (also see later); minor differences in the biological activity of the oligomers may, of course, exist.

No sugar moiety could be detected in the I protein by the conventional techniques. The protein appears to be free of any detectable nuclease or protease activity.

THE I PROTEIN FROM THE ZAH

The isolation of the I protein from ZAH was carried out by both the methods used for isolating this protein from normal rat liver (that is, the Biogel P-200 method and the immunoadsorbant column method). The behaviour of the protein from the ZAH cells on PAGE using various types of gels and both Coomassie Blue and silver for staining, was identical to that of the protein from normal rat liver (Table 1; Figs. 5 and 6). Both the proteins, on silver staining following SDS-PAGE, gave two very close bands (which at times were not separated) in identical positions.

On the immunoadsorbant column, two peaks were obtained as in the case of the protein from normal liver (Fig. 3). Peak I from this column was run on the HPLC gel filtration column wherein five to six fractions were obtained (Fig. 4C; Table 1); these fractions, as in the case of the I protein from normal rat liver, seemed to represent various mixtures of oligomers. The only significant difference seemed to be that one of the oligomers - probably the dimer (band 2 in non-SDS-PAGE of fraction 1 from the HPLC run of antibody-Sepharose column peak I from the ZAH; Table 1) - seemed to represent a substantial proportion of the total protein, the other main component being the monomer. The HPLC fraction 1 (the major fraction) contained predominantly the above-mentioned band 2 (fraction 1a in Fig. 4c consisted exclusively of band 2), whereas in the HPLC fractions 2 and 4, band 1 corresponding to the monomer, was the major constituent (Table 1). As was, therefore, to be expected, on Coomassie Blue staining, non-SDS-PAGE of fraction 1 gave only one band probably corresponding to the dimer (band 2 of Table 1), whereas fraction 2 too gave only one band but with a higher mobility, probably corresponding to the monomer.

The antisera, AS1, raised against the Biogel P-200 peak II from normal liver, which peak appeared to contain all the oligomers of I (although the major component appeared to be the monomer), gave one precipitin band with the HPLC fraction 1a (containing predominantly the dimer), but 2 precipitin bands each with fractions 1b and 2 (which had both the monomer and the dimer in sufficient quantity) from the ZAH; one of the two bands of fraction 1b (probably corresponding to the dimer) was immunologically identical with the single band of fraction 1a and one of the bands of fraction 2 which, between themselves, also showed immunological identity. The other bands of fractions 1b and 2 (probably corresponding to the monomer) also showed immunological identity. The bands corresponding to the monomer and the dimer showed partial cross-reactivity.

Antisera, AS2, raised against a HPLC fraction (Fig. 4A, fraction 2) of the I protein from normal liver, containing almost exclusively the monomeric I, gave single immunologically identical precipitin bands against the HPLC fraction 2 (Fig. 4A,B) of the antibody-Sepharose column peak I, both from normal liver and the ZAH, and the HPLC fraction 1b from the ZAH. Antisera, AS2, however, did not give any precipitin band against the ZAH HPLC fraction 1a (Fig. 4C) consisting almost exclusively of the dimer.

The above observations suggest that there may be antigenic determinants present in certain oligomers (e.g. the dimer) of the I protein from the ZAH, which may not be shared by the monomer, and vice versa. Further, the total I protein as isolated from normal rat liver, is probably immunologically similar but not identical with the total I protein from the ZAH in terms of its oligomeric profile, the protein from the ZAH containing much more of the dimer than the normal I protein.

BIOLOGICAL ACTIVITY OF THE I PROTEIN FROM NORMAL RAT LIVER AND FROM THE ZAH

The crude ICM and peaks I-III eluted from the DEAE-Sephadex A-50 column, peaks II-IV eluted from the Biogel P-200 column, the protein fraction unadsorbed on the immunoadsorbant column and the two protein fractions adsorbed on this column (eluted at pHs 3 and 2.2 respectively), and several of the fractions obtained on HPLC of the two protein peaks from the immunoadsorbant column, were assayed for their biological activity on one or more of the systems already mentioned. Typical results (for normal liver) are given in Tables 2 and 3. The purified I protein or I-containing fractions from normal liver, scored positive in all the assays; the protein appeared to lose a part of its activity after passage through the HPLC column. It seemed to be unstable; for best results, it was necessary to go through the entire purification procedure as quickly as possible and to do the assay immediately afterwards. Generally speaking, maximum inhibitions (70-90%) were obtained with 50-100 µg of the pure protein/ml. A plateau was reached at the above level of inhibition, so that further increase in the concentration of the protein did not lead to any further inhibition. In the case of crude I-containing fractions, a correspondingly higher amount of the protein material was required for equivalent inhibition.

On the basis of the yield of the purified I protein obtained from normal liver, it would appear that this protein represents at least 0.3% of the total protein of rat liver; it would thus appear to be a major liver protein. Since the rates of uptake of amino acids in low-concentration cell suspensions from normal rat liver, or in the cell suspensions from regenerating rat liver, or in the ZAH, are about an order of magnitude higher than the rates obtained in resting liver cells (liver slices or high-density liver cell suspensions from normal adult rat liver), it would appear that the I protein from normal liver reduced the high rate of uptake obtained in the dividing (or, metabolically equivalent) cells to the low level obtained in resting cells. This protein had no effect on the uptake of amino acids in rat liver slices. It also showed no effect on the uptake of amino acids or on DNA synthesis, either in rat kidney cells obtained in suspension as by Thimmappayya et al. (1970) or in the Yoshida ascitic sarcoma. It would thus appear to be tissue-specific.

The I-like protein isolated from the ZAH, even though similar in many respects to the I protein from normal adult rat liver, did not show any biological activity in any of the assay systems tried, using normal liver cells or the ZAH or the Yoshida ascitic sarcoma.

Table 1. Behaviour on PAGE of various I-containing fractions during isolation of the I protein from normal rat liver and the ZAH

Sl. no.[+]	Fraction	Number of bands obtained on: Non-SDS-PAGE[++] (Coomassie Blue stain)	Non-SDS[++] PAGE (silver stain)	Non-SDS gradient PAGE (silver stain)	SDS-PAGE (silver stain)[‡]
1.	DEAE-Sephadex A-50 column adsorbed peak II (Fig. 2)	Many	-	-	Many
2.	Antibody-Sepharose column adsorbed peak I (eluted at pH 3.0) (Fig. 3)	One	Four (1*,2,3,4)	-	-
3.	Antibody-Sepharose column adsorbed peak II (eluted at pH 2.2) (Fig. 3)	One	Three (1*,2,4)	-	-
4.	HPLC of antibody-Sepharose column adsorbed peak I (Fig. 4A):				
	Fraction 2	One	Three (1*,2,3)	Three	One or two
	Fraction 3	-	Three (1*,2,3T)	Two	One or two
	Fraction 4	One	Two (1*,2T)	Two	One or two
5.	HPLC of antibody-Sepharose column adsorbed peak II (Fig. 4B):				
	Fraction 3a	-	Three (1*, others T)	-	One or two
	Fraction 3b	-	-	-	One or two
6.	HPLC of antibody-Sepharose column adsorbed peak I (Fig. 4C):				
	Fraction 1 (a+b)[‡‡]	One (2)	Three (1*,2*,4)	-	-
	Fraction 2	One (1)	Four (1*,2,3,4)	-	One or two
	Fraction 4	-	Two (1*,2)	-	-

Footnotes to Table 1:

\+ 1-5, from normal rat liver; 6, from the ZAH.

++ The bands are numbered, in parentheses, in decreasing order of mobility; the major bands carry an asterisk. T, traces. Silver staining detected minor bands which were not detected by Coomassie Blue staining. The lowest molecular weight band (presumably the monomer) electrophoresed in the same position in every case.

‡ The position of all these bands on the gel appeared to be identical. When two bands were obtained, they were very close to each other; they merged into one when large amounts of protein were loaded on the gel.

‡‡ Fraction 1a (Fig. 4C) consisted exclusively of band 2 (probably the dimer).

Table 2. Effect of the various I-containing fractions from normal rat liver on the total uptake of [^{14}C]lysine by the ZAH cells

The assay was done using 2.5 x 10^6 ZAH cells in 3 ml, essentially as described earlier (Bhargava et al., 1979). For details of various steps of purification, see text and Figs. 2-4. Crude preparations of the I protein inhibited the uptake, in the ZAH cells, of glutamic acid, lysine, proline, phenylalanine, and threonine, but not of isoleucine, leucine, valine and methionine; with the purified I, uptake of only lysine has been so far studied.

Step of purification	Fraction	Protein (mg/ml)	Inhibition of the total uptake of lysine (%)
Crude ICM	-	5.0	47
DEAE-Sephadex A-50 column run	Adsorbed peak I	1.3	78
	Adsorbed peak II	1.1	85
	Adsorbed peak III	1.2	5
Biogel P-200 column run	Peak II	1.2	75
	Peak III	1.4	64
	Peak IV	1.6	68
Immunoadsorbant column run	Unadsorbed protein	0.23	12
	Peak I	0.12	45
	Peak II	0.17	39
Peak I from immunoadsorbant column, run on HPLC	Peak II	0.08	54
	Peak III	0.07	67
	Peak IV	0.07	64
Peak II from immunoadsorbant column, run on HPLC	Peak IIIa	0.05	28
	Peak IIIb	0.04	36

Table 3. Effect of crude ICM from normal rat liver on various assay systems for measurement of I activity

The assays were done essentially as described earlier (Bhargava et al., 1979).

ICM (mg/ml)	Assay system			Inhibition** (%)
	Cell type*	Parameter measured	Precursor used	
2.5	ZAH cells	Total uptake	[^{14}C]lysine	68 (87)
5.0	ZAH cells	Total uptake	[^{3}H]thymidine	70
5.0	ZAH cells	DNA synthesis	[^{3}H]thymidine	93
2.5	Liver cells*	Total uptake	[^{14}C]lysine	60
2.5	Liver cells*	DNA synthesis	[^{3}H]thymidine	61 (83)

* The liver parenchymal cells were from regenerating liver.

** Values in parentheses represent inhibition obtained with pure I (400 µg/ml) prepared by method 1.

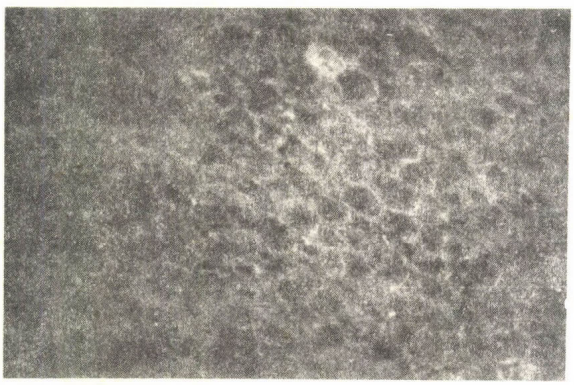

Fig. 7. Localisation of the I protein in rat liver. Only the perimeter of the liver cells showed strong fluorescence. x 1350

LOCALISATION OF THE I PROTEIN

The tissue localisation studies were carried out in collaboration with our colleagues, Drs. S.K. Dubey and P.D. Gupta, using the sandwich immunofluorescence technique. The tissue slices (5-7 microns), frozen or fixed, were treated with antisera (AS1) made in rabbits against the pure I protein (Biogel P-200 column peak 2), and then stained with goat antirabbit IgG-FITC

conjugate; in the control, the slices were treated directly with the second antibody without prior treatment with AS1. These experiments showed clearly and unambiguously, the presence of the I protein at the periphery of the liver cells (Fig. 7); a small fraction of it could also be intracellular. Rat kidney and spleen scored totally negative in this regard, as did embryonic liver. At 24 and 48 h after partial hepatectomy, too, no I protein was found on the periphery of the liver cells, but the protein reappeared at these sites six days after the surgery. Mouse liver, however, scored positive for the I protein, suggesting that it may be more tissue-specific rather than species-specific.

CONCLUSIONS

In conclusion, we have purified to homogeneity a protein which inhibits the uptake of certain amino acids (including several essential amino acids) in both low-density liver cells and in an ascitic liver tumour, and brings down this uptake to the level obtained in high-density or resting liver cells; it has no effect on the uptake of amino acids in the resting cells (e.g., in tissue slices derived from normal adult rat liver). This protein, called I, also inhibits DNA synthesis in liver cells obtained in suspension from a partially hepatectomised animal as well as in the ZAH, and appears to turn over rapidly. Its above-mentioned biological activities seem to be tissue-specific. I thus appears to be a major, liver-specific protein which was not found in the other tissues tested; it also appears to be absent in embryonic and partially hepatectomised livers. It, therefore, satisfies all the criteria for which it has been tested, that had been postulated for the I factor in the model of regulation of cell division and malignant transformation through control of uptake of essential nutrients, put forward earlier (Bhargava, 1977).

A protein which, in many respects, behaves identically to the I protein from normal liver, has also been purified to homogeneity from the ZAH. The ZAH protein is immunologically very similar to the protein from normal liver and has a similar behaviour on fractionation systems such as PAGE and HPLC. It, however, has no biological activity whatsoever as assayed in the various systems mentioned above. As the I protein did not appear to have any sugar moieties, changes in which could lead to a loss of biological activity, the protein obtained from the ZAH could be a mutated form of the normal protein, the mutation leading to a loss of the above-mentioned biological properties, and a change in the pattern of oligomerisation and the stability and/or immunogenicity of certain oligomers, specially the dimer. If this turns out to be so, the isolation of the two proteins reported here would provide substantial support to the model of regulation of cell division and malignant transformation mentioned at the beginning. We are in the process of checking on this point by looking at the peptide maps (and, eventually the sequence) of the I protein from normal liver as well as the I-like protein from the ZAH, using HPLC to separate the peptides.

Another important implication of the model discussed here (Fig. 1) is that, if it is true, there would exist on the cell surface three spatially linked sites: one for the receptor for I; the other through which the transport machinery, at least for essential nutrients, would operate; and the third where the mitogens would bind. It would be possible to determine whether or not this is true, by labelling the receptors for I through a

fluorescent-labelled I protein, and the receptors for a suitable mitogen, differently, and then looking for energy transfer. One could also see whether the two labels cap together under appropriate conditions.

The model also predicts that the sites at which various mitogens that act on the cell surface bind, must overlap as all of them must be spatially linked to the site for the receptor for I. This point could also be checked in the above-mentioned manner.

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DISCUSSION

SCHINDLER:

How do you relate the high molecular weight of your inhibitor molecule to the findings of Holley and Wang that growth inhibitory substances have low molecular weights (≤15 K) and are found in minute amounts?

BHARGAVA:

I am very familiar with the growth-inhibitory factor isolated by Holley and his colleagues at the Salk Institute from dense cultures. This factor acts in extremely small (nanogram) amounts. I am not aware of any studies on the effect of this factor on transport or uptake of essential nutrients, and the mechanism of its action is, I believe, still unknown. It certainly looks different from our factor. In fact, it can be argued that the I factor, if real, will probably be active only in much larger amounts than Holley's factor is.

SCHINDLER:

Do you use reducing conditions for your gel?
Have you proteolyzed the ATPase following its incorporation into phospholipid vesicles?

BHARGAVA:

We have carried out the gel runs both under reducing and non-reducing conditions. Under reducing conditions we obtained two bands for the purified I protein, whereas under non-reducing conditions only one band was obtained. It is not relevant to my talk.

RESCH:

Even the protein purified to homogeneity is required in very high concentration (≅40 µg/ml) to inhibit cellular growth and amino acid transport. How can this be reconciled with a very specific function?
Related to the first question. As the protein you purified apparently is a mass protein in liver, do you have evidence

that it has other regulatory functions than the ones you have described?

BHARGAVA:

As I have already mentioned, we are not surprised that we need fairly high concentrations of the apparently homogeneous I protein for its biological activities. If one takes into consideration the probable number of sites on the cell surface to which it would bind, and assumes a reasonable affinity (not too high and not too low), one can easily see that the minimum concentration at which it would act would be rather high. In fact even before we purified the protein, we could on the basis of the above, rather crude calculations, predict that this protein should account for 0.1 to 1 % of the total liver proteins.
We have so far no evidence that the I protein we have isolated has any other regulatory function than what we have described. This certainly cannot be ruled out and we must consider the possibility that the actual function of this protein in liver may be totally different from what we have ascribed to it. This is always a difficult question to settle in biology.

ZS.-NAGY:

During the recent 4-5 years data have been published demonstrating that the amiloride-sensitive sodium-influx is a necessary prerequisit for the start of mitotic cycle in vivo and in vitro. Why do you neglect all these data in your model? It is quite possible that your protein I plays a regulatory role in the resting sodium permeability of the cell membrane.

BHARGAVA:

It is certainly possible that the I protein plays a role in regulating the ion permeability of the cell membrane. It could indeed be one (or even the main) function of the I protein - a function that may be related to pushing a resting cell into the division cycle.

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MEMBRANE STRUCTURE AND DYNAMICS: FLUORESCENCE DEPOLARIZATION APPROACHES

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INTRODUCTION

The importance of the architecture and fluidity of natural phospholipid bilayer membranes, *i.e.* their structure and dynamics, to the proper functioning of a variety of processes mediated directly or indirectly through intrinsic membrane proteins and their interactions, both mutual and with extrinsic or even further removed partners, may nowadays be regarded as axiomatic. It is a central tenet of the fluid mosaic model of such membranes first mooted in detail in the early 1970's (Singer & Nicolson, 1972). Even neglecting specific lipid-protein interactions, the conformational states of the hydrophobic lipid tails and their flexibility may be expected to influence the conformation of the hydrophobic core of embedded intrinsic proteins. These effects may then be transmitted into their polar regions affecting enzymic and binding properties as well as, *via* the latter, the conformation and functional efficiency of other proteins associated with them. Obviously, any process depending on association equilibria of intrinsic membrane proteins or extrinsic ones bound to them will require appropriate rates of lateral and rotational diffusion of the intrinsic proteins concerned to effect the specific association leading, for example, to formation of a pore *via* dimer or higher multimer association. These rates, particularly the rotational rates, will depend critically on the fluidity of the membrane. Similar considerations will apply also to any specific binding equilibria between small lipophilic molecules, *e.g.* cholesterol, and intrinsic membrane proteins that are necessary to the function of the latter. Again, the density-dependent growth control of normal cells, leading to the establishment and maintenance of tissues and organs, and its abrogation in the malignant state, may be affected by the structure and fluidity of the plasma membrane at which cell-cell interactions take place and whose effects are transmitted without, necessarily, physical transport of material to the interior of the cell.

One reasonable approach out of the gamut of biological, physiological, biochemical and physicochemical approaches currently available in the study of such phenomena with a view to obtaining enough of an understanding of them to be useful - particularly in the diagnosis, biostasis, reversal, cure and eventually prevention of cancers and other clinical manifestations of aberrant cell behaviour - is to compile a detailed comparative physicochemical picture of the structure and dynamics of cell membranes in normal and aberrant states and of their correlation with processes mediated in one way or another *via* membrane proteins. This has involved, and will continue in the future to involve, exhaustive studies of model systems at all levels

of complexity from the simplest pure chemically homogeneous phospholipid monolayers or single bilayer vesicles, through model protein-containing and other small molecule-, *e.g.* cholesterol-,containing phospholipid bilayers or multibilayers, whole membrane fragments and reconstituted systems right up to whole living, viable cells.

The basis of one such physicochemical approach, utilising the fluorescence depolarization of suitable embedded lipophilic probes in the form of fluorescent phospholipid analogues or derivatives, or more commonly small unrelated free aromatic fluorophores, is sketched out in perspective and in rather general terms below. No attempt is made to be comprehensive or to give detailed arguments for some of the statements made or positions taken which, it must be admitted, reflect to at least some extent the personal viewpoint and prejudices of the authors. More exhaustive discussion on some of these points is available in the literature and is referred to as appropriate, along with references to recent comprehensive reviews of the technique of polarized nanosecond single-photon-counting, delayed-coincidence fluorescence spectroscopy and its application in membrane studies.

DEFINITIONS

The fluorescence emitted by organic molecules which have absorption and emission bands in the near ultraviolet and visible range of the spectrum is, in general, polarized. This is the result initially, of *photoselection* in the absorption process. It arises from the fact that the interaction leading to absorption depends, in the cases of most interest here, upon the relative orientation of the electric vector of the linearly polarized, excitation beam and the transition moment vector associated with the absorption band. The memory of this photoselection is carried over into emission *via* the specific orientation of the transition moment vector associated with the emission band relative to the absorption vector, and the fact that the emitted light is characterised by an electric vector lying in the plane containing the emission transition moment vector and the propagation direction and has an intensity which depends on their mutual orientation. Provided that thermal (Brownian) motion or other phenomena such as energy migration between differently oriented fluorophores, the latter of which occurs to an appreciable extent only when the fluorophore is present at high concentration, does not effectively orientationally randomize the photoselected excited-state population before emission takes place (excited-state decay times usually a few to a few tens of nanoseconds), the emission of an initially orientationally completely random ensemble of unexcited fluorophores can exhibit quite high positive or negative polarizations after excitation with linearly polarized light. Formally this phenomenon is most conveniently expressed in the notation introduced by Jablonski (1960):

$$r = (I_{||}-I_{\perp})/(I_{||}+2I_{\perp}) \qquad (1)$$

where r is known as the *emission anisotropy* (EA) and $I_{||}$, $I_{\perp}$ are intensities of components of emission polarized in parallel and perpendicular directions respectively to the electric vector of excitation and measured in the plane normal to it, usually at right angles to the direction of the excitation beam; the denominator, $I_{||}+2I_{\perp}$, is proportional to the total intensity emitted in all directions. Experimentally it is usually necessary to correct the measured intensities for differences in the efficiency of detection of orthogonally polarized emissions or differences in intensity of orthogonally polarized excitation. In practise this is accomplished by utilising the fact that, due to symmetry considerations in such a system, all polarized components of emission measured along a direction parallel

to the electric vector of excitation are equal, leading to:

$$(I_{\parallel}/I_{\perp}) = (I_{VV}.I_{HH})/(I_{VH}.I_{HV}) \qquad (2)$$

in which the polarized intensities on the right-hand side of the equation are those observed experimentally for combinations of vertical (V) and horizontal (H) polarizations of excitation and emission in a laboratory Cartesian frame observing the emission at right angles to the excitation in the horizontal plane.

Both positive and negative EA values are possible: $0.4 \geq r \geq -0.2$, and these limits, which apply for both non-coherent and coherent excitation of low enough intensity that non-linear optical effects are negligible, are approached for rigid or highly viscous samples where reorientational randomization by Brownian rotation and excitation energy transfer during the excited-state lifetime is negligible and the absorption and emission transition moments are close to parallel or perpendicular respectively. Including any inherent depolarizing effects due to possible degeneracy or tri-dimensionality of transitions or torsional vibrations, all approximated as axially symmetric about some mean absorption and emission transition moment vectors $\vec{a}$, $\vec{e}$ and occurring on a much faster time-scale than emission, this may be expressed as:

$$r_0 = 0.4\langle d_a\rangle[(3/2)\cos^2\theta_{ae}-(1/2)]\langle d_e\rangle \qquad (3)$$

where $\langle d\rangle = (3/2)\langle\cos^2\theta\rangle-(1/2)$ are the depolarization factors inherent in the absorption and emission transitions averaged over all angles θ_a, θ_e relative to the mean transition moments $\vec{a}$ and $\vec{e}$ respectively which make an angle θ_{ae} with each other, while the factor 0.4 arises from the initial process of photoselection from the random ensemble.

FLUORESCENCE DEPOLARIZATION AND BROWNIAN ROTATION

In the simplest cases, the evolution of the EA due to Brownian rotation following an instantaneous excitation is a first order process:

$$r(t) = r_0\exp[-t/\phi] \qquad (4)$$

in which r_0 is the EA at time zero (on the nanosecond time-scale) given by Eq.(3), and ϕ is known as the correlation time for the depolarization, while the evolution of the excited-state population registered by the time-dependence of the emission intensity integrated over all directions in space is also first order:

$$I(t) = I_0\exp[-t/\tau] \qquad (5)$$

I_0 representing the intensity at time zero, τ being known as the excited-state lifetime. In such cases, remembering that $I \propto (I_{\parallel}+2I_{\perp})$ and applying Eq. (1), the time-averaged EA, $\langle r\rangle$, measured under conditions of constant illumination intensity is given by:

$$\langle r\rangle = \int_0^{\infty} I(t)r(t)dt / \int_0^{\infty} I(t)dt = r_0[\phi/(\tau+\phi)] \qquad (6)$$

Eq.(6) will apply to any isotropic, *i.e.* spherically symmetric, rotor in an isotropic medium, and also, in such a medium, to any cylindrically symmetric rotor in which either or both $\vec{a}$ and $\vec{e}$ lie along the symmetry axis.

Some fifty years ago, the above correlation times for spherical rotors and for ellipsoids of revolution had been related by Perrin (1926, 1936) to their size and shape and to the viscosity of the isotropic bathing medium under the implicit assumption of a sticking boundary condition, leading in the reciprocal form of Eq.(6) - Perrin's law of isotropic depolarization - to:

$$r_0/\langle r\rangle = 1 + f(kT/\eta v) \qquad (7)$$

where k is the Boltzmann constant, T the absolute temperature, v the effective molecular volume (including any possible complexed solvent or solvent "shell"), η the viscosity of the medium, and f a shape factor which is unity for the spherical rotor and related to the axial ratio ρ of a prolate ellipsoid of revolution ($\rho>1$) by:

$$f = 3\rho[(2\rho^2-1)(\rho^2-1)^{-1/2}\ln\{\rho+(\rho^2-1)^{1/2}\}-\rho]/2(\rho^4-1) \qquad (8a)$$

and of an oblate ellipsoid of revolution ($\rho<1$) by:

$$f = 3\rho[(2\rho^2-1)(1-\rho^2)^{-1/2}\arctan\{(1-\rho^2)^{1/2}/\rho\}-\rho]/2(\rho^4-1) \qquad (8b)$$

Details of the technique of polarized nanosecond fluorometry and its application in membrane systems may be found in a number of recent reviews (Wahl, 1975; Badea and Brand, 1979; Dale, 1983).

THE "MICROVISCOSITY" CONCEPT

In some early experimental studies which followed this work, it was found that small fluorophores dissolved in a variety of gel systems which have extremely high viscosities as measured by the usual macroscopic methods, nevertheless exhibited high degrees of depolarization, unless they were actually adsorbed to the gel-forming material itself, indicating a local viscosity ("microviscosity") for the fluorophoric probe much closer to that of the pure solvent than to the global viscosity ("macroviscosity") of the gel as a whole (Pringsheim, 1949). More recently this concept of "microviscosity" as signalled by the fluorescence depolarization of a suitable probe embedded specifically in a microenvironment of interest has been taken-up and widely exploited in the examination of a broad range of model and biological membrane systems, including whole cells, following the seminal contributions of Shinitzky and his school (for reviews of this approach see Shinitzky & Barenholz, 1978, and Shinitzky & Yuli, 1982).

These studies have utilized almost exclusively the elongated aromatic fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH), many of whose spectroscopic characteristics (absorption and emission spectra, absorption coefficient, excited-state lifetime and quantum yield) and its facile, quantitative introduction into the membrane phospholipid bilayer interior from suitably prepared non-fluorescent aqueous microcrystalline suspensions, appeared to render it close to ideal for this purpose. In analogy with Eq.(7), the "microviscosity" of the interior of bilayer membranes is assessed with DPH using:

$$r_0/\langle r\rangle = 1 + C(\langle r\rangle)\frac{T\tau}{\eta} \qquad (9)$$

where $C(\langle r\rangle)$, initially held to be constant but later given as a function of $\langle r\rangle$ (Shinitzky & Barenholz, 1978), was determined using an oil of "appropriate" macroviscosity as a calibration standard. The main assumptions inherent in this approach are:

(i) that the complex anisotropic microscopic motions of this plate-like molecule, including the possibility of "slip" in its plane, in a solvent whose molecules are of comparable dimensions, directly reflect the macroscopically measured viscosity - an assumption belied by the observation that different oils give rise to different curves;

(ii) that the interior of the bilayer is not more structured than is the calibration oil, *i.e.* that it is isotropic, exhibiting no order persistent over the nanosecond time-scale during which the EA is registered - an assumption already negated before the advent of the

method by observations on the polarized fluorescence of similarly shaped fluorophores in oriented membranes, which showed that the molecules were indeed ordered in the membrane environment;

(iii) that the bilayer environment is homogeneous, both transversely and laterally, in respect to both its "microviscosity" (admittedly, only an average viscosity is claimed for the method) and its excited-state decay, the latter being assumed not only homogeneous, but also first order, this also being demonstrably invalid in many, if not indeed all, bilayer membrane systems.

Detailed discussions of these and related points have been presented elsewhere (Dale, 1983; Zannoni *et al.*, 1983).

TIME-RESOLVED FLUORESCENCE DEPOLARIZATION

The advent of polarized nanosecond time-resolved fluorometry has enabled a more direct examination of the degree of order imposed on fluorophores in such media, even when the overall orientational distribution is isotropic by virtue of the random orientation of anisotropic membrane fragments, or of single- or multi-bilayer vesicles, in suspension. If there are constrains on the reorientation of the fluorophore within the bilayer environment, it is expected that the initially highly polarized, photoselected excited-state fluorophore population will never (on the time-scale of the emission process, during which there will be negligible reorientation of the membrane fragments or vesicles themselves) relax to an isotropic orientational distribution, *i.e.* there will remain a residual non-zero EA, conventionally designated r_∞, at long times.

As the simplest illustration of what might in various circumstances occur, the model to which DPH has often been approximated, that of an elongated cylindrical rod or prolate ellipsoid of revolution with one or both $\vec{a}$ and $\vec{e}$ directed along the long axis, may be considered. In an ordinary (*i.e.* isotropic) solution the EA decay of such a molecule is monoexponential following Eq.(4). Three possibilities then exist, *a priori*, for the EA decay of such a probe embedded in a membrane bilayer interior:

(a) monoexponential, reflecting at one extreme an isotropic fluid environment;

(b) immediate, unobservable (on the nanosecond time-scale) EA decay to a constant value r_∞ smaller than r_0, reflecting at the other extreme a palisade or picket-fence kind of bilayer structure, the fluorophore being free to move without significant viscous opposition within a limited orientational range bounded by an impenetrable wall;

(c) decay of the EA on the nanosecond time-scale from its initial value r_0 to a smaller plateau value r_∞ at long times, reflecting both viscous opposition to reorientation and a non-isotropic equilibrium distribution of the probe in both ground and excited states.

For DPH in phospholipid membrane systems, it is always the last possibility described above that is observed qualitatively, and the EA decay is representable by:

$$r(t) = (r_0 - r_\infty)F(t) + r_\infty \qquad (10)$$

In general, F(t) may be expected to be a complex function. It has been derived explicitly for only two reasonable models of restricted reorientation of a cylindrical rod: "wobbling" or "random walk" (i) within a defined cone (Kinosita *et al.*, 1977) or (ii) over a truncated Gaussian distribution (Kinosita *et al.*, 1982). The result is indeed complex,

comprising an infinite series of exponential terms with, however, quite rapidly decreasing pre-exponential coefficients. The validity in practise of the proposed approximation of Eq.(11) by:

$$r(t) = (r_0-r_\infty)\exp[-t/\phi] + r_\infty \qquad (11)$$

at least in the first of these models, has been discussed elsewhere (Dale, 1983). The ϕ value thus determined depends not only on the "true" correlation time (which would be obtained directly as $r_\infty \to 0$) but also on the magnitude of the cone angle or width of the distribution. The latter alone determines r_∞ and can be estimated from it, the former requiring both the "observed" ϕ and r_∞ for its evaluation within the framework of the model employed. However, since it has been shown on general grounds for such distributions that the initial slope of the normalized EA, $r(t)/r_0$, should supply the true correlation time directly, then a statistically adequate fit of the data employing an arbitrary multiexponential function for F(t):

$$r(t) = \sum_{i=1}^{N} \beta_i \exp[-t/\phi_i] + r_\infty \qquad (12)$$

should yield this value as the reciprocal of the weighted harmonic mean correlation time:

$$\phi_{true} = \left[\sum_{i=1}^{N} (\beta_i/\phi_i)/r_0\right]^{-1} \qquad (13)$$

(Kinosita *et al.*, 1977). The advantage of such a determination, provided that r_0 has a reasonable value, *i.e.* that it is not lower than expected from measurements made in viscous isotropic solvents due to sub-nanosecond reorientational relaxation, is that it is essentially model independent. In addition, while r_∞ can be related also to the geometrical parameters of some orientational equilibrium distribution model, it is most simply expressed in a model-independent way *via* an order parameter S for the (radially symmetric) distribution:

$$r_\infty = S^2 \qquad (14)$$

(Heyn, 1979; Jähnig, 1979), where $S=(3/2)\langle\cos^2\psi\rangle-(1/2)$, ψ being the angle between the axis of the rod and the axis of its radially symmetric orientational distribution, averaged over all possible orientations weighted for their probability. As will be indicated later, this may in some cases, possibly in the majority, represent an oversimplification in assuming that the order parameters for ground and excited states of the probe are identical.

STEADY-STATE FLUORESCENCE DEPOLARIZATION

If the decay of emission intensity is well approximated by Eq.(5) and Eq.(11) also represents a good approximation to the time-dependence of the EA, an expression for the steady-state (time-averaged) EA is readily derived *via* the integrations of Eq.(6) and leads to the equivalent, for restricted rotation, of Perrin's law of isotropic depolarization:

$$(r_0-r_\infty)/(\langle r\rangle-r_\infty) = 1 + (\tau/\phi) \qquad (15)$$

Lifetime-resolved emission anisotropy measurements in which $\langle r\rangle$ is measured as a function of τ, which is varied by quenching the fluorescence with oxygen at high pressures, affords a neat solution to the analysis of this relationship (Lakowicz *et al.*, 1979; Lakowicz & Knutson, 1980). If the (first-order) lifetime is known at one pressure, it can even be estimated at others by simple intensity comparisons *via* the relationship:

$$\tau_1/\tau_2 = I_1/I_2 \qquad (16)$$

Alternatively use might be made of one or other of the linear correlations between r_∞ and $\langle r \rangle$:

$$r_\infty = A\langle r \rangle - B \qquad (17)$$

found for DPH embedded in a range of membrane systems, both model and natural (Jähnig, 1979; Hildenbrand & Nicolau, 1979; Fulford & Peel, 1980; van Blitterswijk *et al.*, 1981), to obtain at least some approximate structural information. Since these relationships appear to rely on the approximate constancy of the factor (τ/ϕ), dynamic information is also obtainable given an estimate of τ. The interpretation of r_∞ as an indicator of membrane *fluidity* rather than *order* (van Blitterswijk *et al.*, 1981) would seem to be in some conflict with the orthodox view of membrane structure and dynamics as monitored by fluorescence depolarization and embodied in the theoretical results summarized above.

In view of the approximations inherent in both the derivation of Eq. (15) and, possibly as or more importantly, in its application, particularly *via* Eq.(17) - estimates of τ, ϕ and r_∞ are all uncertain averages which will vary depending on the precise method of their derivation - neither of the steady-state approaches described above is capable of affording as detailed or accurate a picture of structure and dynamics as may be obtained by time-resolved depolarization methods, and even orthodox estimates of order and fluidity thus obtained should be viewed with caution.

FLUORESCENCE DEPOLARIZATION IN ORIENTED MEMBRANE SYSTEMS

It has, of course, been recognized for some time that a reasonably complete realistic description of the depolarization process in anisotropic media could only be expected to be obtained from oriented samples. The earlier work - both experimental and theoretical - concentrated, for practical reasons, on steady-state measurements and a few selected experimental geometries, and even the more recent theoretical work, including descriptions of the time-dependence of the depolarization process, has by and large not concerned itself with generalizing to arbitrary orientations (see review of Zannoni *et al.*, 1983). Specific consideration has not been given either to the exact amount of information it is possible to extract from such experiments or to the optimal method of obtaining it. Both these questions have recently been answered by applying the elegant methods introduced by Zannoni (1979a, b; 1981) to the general case of completely arbitrary experimental geometry, introducing appropriate sample and probe symmetries (Dale and Marszalek, *in preparation*). The result may be written:

$$\begin{aligned} I_{EE'}(t) = \frac{I(t)}{9}[1 &+ (3\cos^2\theta_E - 1)U_0 + (3\cos^2\theta_{E'} - 1)V_0^*(t) \\ &+ (3\cos^2\theta_E - 1)(3\cos^2\theta_{E'} - 1)W_{00}(t) \\ &+ 12(\sin\theta_E\cos\theta_E)(\sin\theta_{E'}\cos\theta_{E'})\cos\xi\ W_{11}(t) \\ &+ 3\sin^2\theta_E\sin^2\theta_{E'}(2\cos^2\xi - 1)W_{22}(t)] \end{aligned} \qquad (18)$$

for a linearly polarized component $I_{EE'}(t)$ - the subscripts identifying the electric vectors of linearly polarized excitation $\vec{E}$ and emission $\vec{E}'$ - of the total emission I(t), where θ_E is the angle between $\vec{E}$ and the director of the fluorophore distribution, which is coincident with the normal to the bilayer in lipid membrane systems, $\theta_{E'}$ that between $\vec{E}'$ and the director, and ξ the azimuth between them in the plane of the membrane, perpendicular to the director. The quantities U_0, $V_0^*(t)$, $W_{00}(t)$, $W_{11}(t)$ and $W_{22}(t)$ contain information about the disposition of $\vec{a}$ and/or $\vec{e}$ within the molecular framework of the fluorophore, about the distribution of the fluorophore itself within the anisotropic sample and also about its dynamics. The

order parameters contained in U_0 and $V_0^*(0)$ are identical and refer to the ground-state equilibrium distribution of the fluorophore; $V_0^*(t)$ is time-dependent if the ground- and excited-state equilibrium distributions are different. $W_{00}(t)$, $W_{11}(t)$ and $W_{22}(t)$ contain orientational correlation functions, that of $W_{00}(t)$ being of the same form as the EA in Eq.(10), *i.e.* having a time-dependent and a time-independent part. An equation identical in form with Eq.(18) has appeared recently in the literature (van der Meer *et al.*, 1982) but was developed and interpreted directly in terms of the distributions of $\vec{a}$ and $\vec{e}$ rather than of the molecular frame in which they are embedded.

Provided that a suitable set of experimental geometries is employed, a total of up to seven distribution and molecular parameters are obtainable from $I_{EE'}(0)$ and $I_{EE'}(\infty)$. They comprise second rank order parameters for ground- and excited-state molecular equilibrium orientational distributions, fourth rank order parameters for the ground state, and up to four of the molecular constants describing the orientations of $\vec{a}$ and $\vec{e}$ within the fluorophore. In general, more than seven parameters contribute to $I_{EE'}(t)$, even in the case of the simplest approximations to molecular shapes, *i.e.* rods, discs and ellipsoids of revolution. However, if the fluorophore itself is the molecular unit of concern, there is usually enough information available about the orientations of the transitions in the molecular frame to obviate this. Thus, treating DPH, for instance, as a cylindrical rod or prolate ellipsoid of revolution with $\vec{a}$, $\vec{e}$ and the principal molecular axis coplanar, the second and fourth rank order parameters for the ground state equilibrium orientational distribution of the axis, the second rank order parameter for its excited state distribution, θ_a, θ_e, $\langle d_a \rangle$ and $\langle d_e \rangle$ are all obtainable. For platelike molecules such as perylene, for instance, with $\vec{a}$ and $\vec{e}$ lying in the molecular plane, order parameters for the restricted orientational distribution of the axis normal to the plane, ($\theta_a = \theta_e = \pi/2$) and also axes in the plane may be simultaneously determinable, *i.e.* measures of the restrictions on reorientation both in-plane and out-of-plane may be available. A full description will be presented elsewhere (Dale and Marszalek, *in preparation*).

The information obtained on orientational distributions by studying fluorescence depolarization in oriented systems is by no means complete. It is sufficient, however, to eliminate ambiguity as to the identity of ground- and excited-state equilibrium molecular orientational distributions with a reasonable degree of certainty. It also imposes acceptability criteria for proposed distribution models: they must be consistent with both the second *and* fourth rank order parameters for the ground-state distribution, and would be expected to retain their consistency when the system undergoes small perturbations, such as induced, for instance, by changing the temperature or pressure over a range in which no phase separation of components of the system or phase transition occurs.

The time-dependencies $V_0^*(t)$, $W_{00}(t)$, $W_{11}(t)$ and $W_{22}(t)$ are all experimentally determinable, and are also theoretically derivable from the form of the potential field which generates the orientational distribution, but with the added ambiguity of choice between models for the diffusional process itself (see *e.g.* Zannoni, 1981). They may, or may not, therefore, prove useful in more finely checking the consistency of orientational distribution models. Obviously, information about effective viscous opposition to rotation is also contained in these time-dependencies. However, it seems likely in view of the structuredness of the membrane interior, that this will be a *vector* quantity, not a *scaler* one as in normal isotropic fluids, *i.e.* the viscous opposition to a particular rotational mode of the probe will depend, not only on its transverse location in a "viscosity gradient" across the membrane, but also on the direction in

which the rotation is occurring, *e.g.* parallel or perpendicular to the membrane plane. Interpretation of apparent coefficients quantitatively in terms of viscosity, or even qualitatively as a rough- and ready- relative fluidity indicator, would seem at this stage to warrant the exercising of more than a modicum of caution, even in chemically homogeneous membrane systems. In heterogeneous model membranes, and even more so in natural membranes, not only the above factors of vectorial viscosity and transverse heterogeneity have to be considered, but also lateral heterogeneity and, indeed, the possibility of specific interaction of the probe with particular membrane components.

A great deal of both theoretical and experimental effort at all levels of membrane complexity is still required if these uncertainties are to be unravelled, and perhaps allow a detailed enough understanding of the architecture and fluidity of biological membranes to be of real value in delineating their role in the normal functioning of membrane-associated processes and in their pathological dysfunction.

ACKNOWLEDGEMENTS

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DISCUSSION

MONTECUCCO:

Considering the X-ray and BLM data on the localization of alkanes in lipid bilayers, would you think that DPH is also localized in the middle of the bilayer, with its long axis in the plane of the membrane? In this respect would it be better to use parinaric acid containing lecithin or DPH containing lecithin?

DALE:

DPH is probably predominantly localized near the middle of the bilayer. Its long axis tends to lie perpendicularly to the membrane plane as shown by fluorescence polarization measurements in oriented multibilayers. Orientation of part of the DPH parallel to the bilayer plane is not completely ruled out, but it may only be a small fraction. The use of parinaric acid, parinaric acid-containing lecithin or DPH-containing lecithin should remove some of this uncertainty since, being attached at one end near to the polar head group, they will be localized in terms of their depth within the bilayer, and restricted in their reorientation about the attachment site to the glycerol backbone.

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IgGFc RECEPTOR POLYMERISATION; ITS EFFECT ON BINDING SITE SPECIFICITY AND FUNCTION

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Fc receptors are immunoglobulin binding structures on the surface of various cells interacting with the Fc part of different classes and subclasses of antibody molecules. Their heterogeneity in binding specificity as well as in functions mediated by these structures reflect the structural heterogeneity of the antibody binding receptor molecules.

The heterogeneity of receptors may mean either that

- the different binding specificity of IgG binding receptors is the function of molecules differing in their primary structure, or

- the binding specificity of different conformational forms of the same molecule is different.

The class and subclass specificity of Fc receptors on various cells is the consequence of the former type of Fc receptor heterogeneity. On the other hand, we described another form of heterogeneity of IgG binding Fc receptors (FcR) on peripheral mononuclear cells which depends on the differing conformational forms of the same molecules. We have found, namely, that the monomeric form of these receptors possesses only one active binding site with a CH3 domain specificity, and due to possible conformational changes, following the polymerisation of the receptors a second active binding site is expressed which interacts with the CH2 domain of the IgG molecule [Gergely et al. 1982].

The observation that a population of the peripheral human mononuclear cells shed their IgG Fc receptors (FcR) following the 4-37°C temperature shift in contrast to another population which does not shed the FcR under identical experimental conditions called our attention to the existence of different types of the IgG binding structures on these cells [Sándor et al. 1979]. The mobile or free form of receptors (called FcRI) shed from the cell surface and can be recovered from the supernatant of the cells treated by temperature shift. The immobile receptors (called FcRII) do not shed following the

temperature shift [Sándor 1979]. The isolated shed FcRI molecules were characterized and compared with the immobile counterparts.

The FcRI molecules turned out to be monomeric molecules with a molecular weight of 60.000 d. They bind to the Fc part of complexed IgG, their binding, however, does not interfere with that of the immobile FcRII molecules. We found namely that the interaction of the soluble FcRI molecules with sensitized erythrocytes (EA cells) does not inhibit the EA-rosette formation of FcRII bearing cells. The FcRI molecules do not agglutinate EA cells and do not inhibit complement dependent lysis of the sensitized red blood cells. On the other hand, the binding of C1 to the EA cells interferes with the EA rosette formation via FcRII but not with that of FcRI positive cells. Based on these observations we concluded that

- the soluble form of the mobile FcRI molecules is monomeric,

- the binding sites of the FcRI and FcRII molecules on the IgG Fc are not identical,

- the binding site of FcRI is not on the C1-binding CH2 domain, in contrast to that of FcRII which is either on or close to this site.

Since several cell surface components are capable of interacting with cytoskeletal structures [Schlessinger 1983] or with each other [Schlessinger 1980], the question was raised whether this form of FcR heterogeneity could be the consequence of such molecular interactions. This would mean that the interaction of the monomeric FcRI molecules with cytoskeletal proteins [or their muscular counterparts] or with each other may result in the

- immobilization of the receptors, and in

- alteration of their binding specificity.

This hypothesis was controlled in various studies.

To control the possible interaction of shed FcRI molecules and muscle proteins the interaction of shed FcRI molecules with actin, myosin and actomyosin was investigated. Neither actin nor myosin absorbed the shed FcRI molecules, in contrast to actomyosin which quantitatively absorbed the soluble receptors from the supernatants of the shed cells. Studying the binding properties of the actomyosin-FcRI complexes we found that the absorbed (crosslinked) FcRI molecules on the actomyosin surface inhibited not only the rosette formation of the FcRI bearing cells but that of FcRII positive cells as well, i.e. the crosslinked FcRI molecules behaved like FcRII [Uher et al. 1981] (Fig. 1).

Transglutaminase, a Ca^2 dependent intracellular enzyme which catalyzes the formation of ε-(γ-glutamyl)lysine crosslinks was also used to crosslink isolated soluble FcRI molecules

[Fésüs et al. 1982]. This form of FcR crosslinking resulted in similar alterations in binding properties as we found in the case of actomyosin-FcRI complexes. Namely, the transglutaminase crosslinked FcRI molecules behaved in rosette formation inhibition experiments like FcRII molecules (Fig. 2).

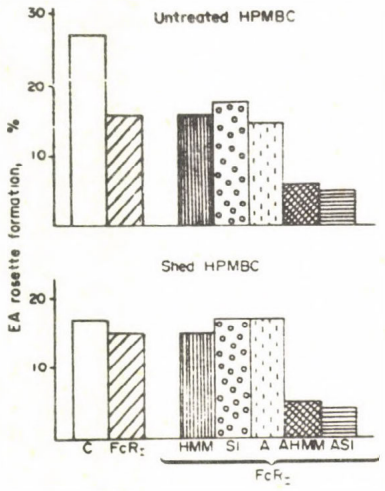

Fig.1. EA rosette inhibition by unabsorbed and absorbed supernatants of human peripheral mononuclear blood cells (HPMBC); C control; HMM. heavy meromyosin; S1. proteolytic fragment of myosin containing the ATPase-active center and the actin binding site: A. actin.

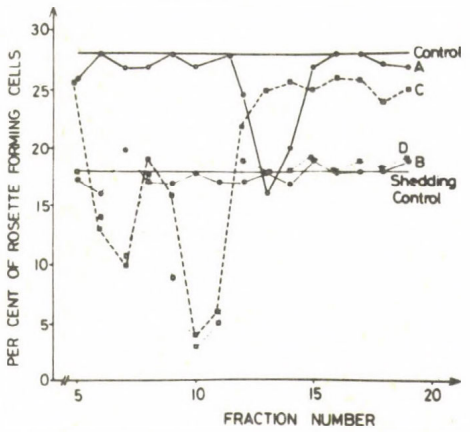

Fig.2. Gel filtration patterns of FcR_1 on Sephadex G-150 as revealed by the inhibition of EA rosette formation of nonshed (control line) and shed (shedding control line) PMBC. A: FcR_1 inhibition of EA rosette formation of control PMBC; B: FcR_1 inhibition of EA rosette formation of shed PMBC; C: TGase-treated FcR_1 inhibition of EA rosette formation of control PMBC; D: TGase-treated FcR_1 inhibition of EA rosette formation of shed PMBC.

Using Facb fragments of rabbit IgG antibodies for sensitization of erythrocytes and isolated labelled $(CH3)_2$ domains we could give a detailed mapping of the FcR binding sites. Since in these studies only the presence of actomyosin bound FcRI molecules were capable of mediating the binding of the iodine labelled CH3 domains to the Facb-sensitized erythrocytes, we concluded that

- the FcRI molecules possess only one active binding site interacting with the CH3 domain of the IgGFc,

- the crosslinking of the FcRI molecules results in the expression of a second active binding site besides the pre-existing CH3 specific one which binds to the CH2 domain.

The expression of this second binding site may be the consequence of conformational alterations which appear following the crosslinking (polymerisation) of the monomeric receptor molecules. One cannot exclude, however, the possibility

that the CH2 specific binding site is expressed on the monomeric form as well, but its binding affinity is too low and the inter- or/and intramolecular alterations due to the cross-linking increase the binding affinity of this site.

The possibility of FcR conversion on the cell surface was also controlled and we found that both FcRI to FcRII and FcrII to FcRI conversion can be induced.

The experiment demonstrated in Fig. 3 shows that ligand binding to FcR-s results in FcRI-to FcRII conversion. The binding of immune complexes to the peripheral mononuclear cells does not change the shedding capacity of the FcRI when the incubation time is shorter than 10 minutes. Longer interaction of the ligand with the FcR bearing cells results in a long lasting FcRI to FcRII conversion, i.e. the temperature shift does not result in shedding.

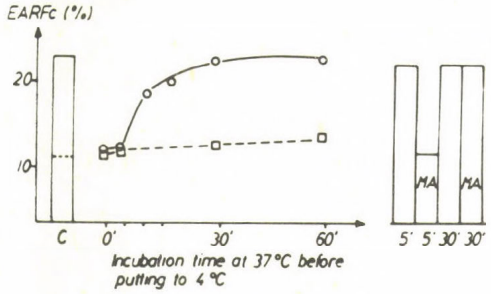

Fig.3. The effect of pre-rosetting time on the shedding of EA rosettes. EA rosettes were preincubated at 37°C in the presence, □---□ , and in the absence, O——O, of 30 mM methylamine before putting them to 4°C for 1 h. Then the EA rosette values were determined after re-incubating at 37°C for 45 min. One representative out of 7 experiments is shown with the control PMBC rosette values before (C——) and after (C---) a temperature shift. On the right EA rosette values of the same donors are shown when the 30 mM methylamine was added to the rosettes at the 5th and 30th minutes of the first 37°C incubation.

It was found by us earlier that the binding of some anti-B2-microglobulin antibodies to the lymphocytes membrane results in coshedding of the FcR-s and B2-microglobulin-anti-B2-microglobulin complexes [Sármay et al. 19]. It turned out that following the binding of these antibodies both the mobile and immobile FcR molecules shed from the membrane, i.e. the crosslinked and/or anchored FcR molecules are also released following the membrane reordering induced by the antibody. It was also found that the shed receptors recovered from the supernatant of the cells have the characteristics of the mono-

meric soluble FcR-s (FcRI). Due to the membrane reordering induced by the anti-B2-microglobulin antibodies an FcRII to FcRI conversion takes place.

It seems that the interaction of FcR-s with other cellular components alters dynamically during the cell cycle. It is well known that the expression of FcR-s on T cell membrane is one of the consequences of ConA stimulation. Controlling the effect of anti-B2-microglobulin antibodies on the newly expressed FcR-s on ConA stimulated cells we found that the FcR-releasing effect of the antibody is totally abolished in the early phase of stimulation (Fig. 4). Later, the typical releasing effect of the anti-B2-microglobulin antibodies could be observed again [Sármay et al.]. This also points to possible functional consequences of the FcR polymerisation on the cell surface.

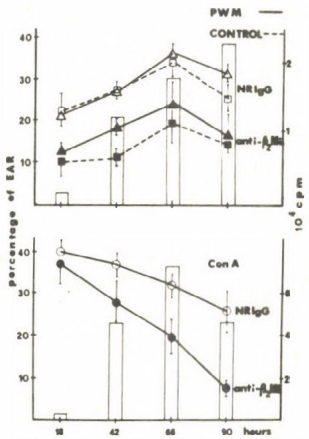

Fig.4. Alteration in the interrelationship between FcR and B2Mi during the activation with PWM or Con A. The symbols represent the averages of EAR formed by the lymphocytes of six different donors after treatment with normal rabbit IgG (NRIgG) (open symbols) or anti-B2Mi (closed symbols). The columns show the rate of [^{3}H]thymidine uptake in parallel samples.

The functional importance of this type of FcR heterogeneity was studied by us in the antibody dependent cell mediated effect/or function of FcR bearing killer cells. The active role mediating the killing signals was proved in this system where the antibody-sensitized cells are the targets of the FcR bearing effector cells. Characterizing the FcR-s of the effector lymphocytes in ADCC we found that these cells belong to the FcRII positive population, i.e. they bear the polymerized form of the IgG binding receptors, interacting both with the CH2 and CH3 domains of the sensitizing antibody. We could also prove that the prerequisite of this killing is the binding through both binding sites. If one of the latter was inhibited in the interaction with the corresponding domain the effectivity of the killing significantly de-

creased. The first component of the complement system (C1) which binds to the CH2 domain as well as soluble FcRI, interacting with the CH3 domain dose dependently inhibited the killing. Similar results were found when synthetic peptides representing linear sequences of the CH2 domain were used.

Based on our observations we suggest that the two forms of FcR-s as well as the dynamic alteration from the monomeric form to the polymeric and vice versa are of functional importance:

- The two forms of receptors represent binding structures with different

  · binding affinity
  · valency
  · binding specificity

resulting in different discriminatory capacities,

- The alteration of receptor polymerisation (clustering) during the cell cycle may reflect an altered functional capacity of the cells and may mediate different signals to the cells resulting in different functions of the same cell.

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DISCUSSION

RESCH:

Your original experiments show that $Fc\gamma R_I$ and $Fc\gamma R_{II}$ are expressed on different cells. Could you elaborate on the nature of these cells - are they subpopulations or do they represent e.g. different functional states of identical populations?

GERGELY:

Both types of receptors can be found in every lymphocyte subpopulation and on monocytes. Keeping in mind that $Fc\gamma R_I$ can be transformed to $Fc\gamma R_{II}$ on the very same cell, one can say that it is a functional marker of the same population. However, in the sense that some cell-types, like macrophages, have only the $Fc\gamma R_{II}$ type of $Fc\gamma$ receptors one can say that this heterogeneity represents a subpopulation marker.

TRÓN:

If I have understood well, it is possible to induce the $Fc_I \rightarrow Fc_{II}$ transition simply by incubating the cells at $37^{\circ}C$ in the absence of antibodies. My question is: what happens if you incubate the cells at a lower (say at $10^{\circ}C$) temperature, would it induce a receptor polymerisation? Regarding the mechanism of the receptor aggregation: have you tried to make these experiments using cytoskeleton disrupting agents? In case you assume that the aggregation is a simple polymerisation process, I think you have to allow in your model a second aggregation site as well.

GERGELY:

$Fc\gamma R_I \rightarrow Fc\gamma R_{II}$ transition can happen due to extensive $Fc\gamma R$-crosslinking by polivalent ligands. That can be inhibited by low temperature. As one part of the $Fc\gamma R^+$ cells have preclustered receptors we suggest, that non-$Fc\gamma R$ mediated signals - like differentiation, mitogenic stimuli -

can induce the receptor-transition as well. At this stage we do not have enough data to speak about the nature of the receptor aggregation of the latter mechanism.

BHARGAVA:

What is the actual quantitative difference between the affinities of the two types of receptors for IgG?
Is there any cross reactivity of any of the two receptor forms with IgM? And are then, similarly, two types of receptors for IgM, too?

GERGELY:

Using panels of erythrocytes coated with different amount of IgG (EA) we had shown the higher cellular avidity of $FcRII^+$ cells. FcRII cells bind EA sensitized with a small amount of IgG, while $FcRI^+$ cells bind EA coated with a high amount of IgG.
We published data (Uher, 1980. Cell. Immunol.) about a similar heterogeneity of IgMFcRs. We have shown the existence of an apparently monomeric and a polymeric form of the IgMFcR. The two forms had different avidity, and anchorage, but not like in the IgGFcR system, both types of IgGMFcR had similar binding specificity for the IgM.

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DYNAMIC AND PHYSICAL PROPERTIES OF INNER AND OUTER NUCLEAR MEMBRANE

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The cell nucleus is encapsulated by perhaps the most dynamic membrane system in biology, yet little is known about the fluid dynamics of nuclear membrane (Wunderlich et al., 1976; Gonzalez and Kasper, 1981; Morré et al., 1979). No systematic analysis of the mobility of proteins and lipids in the nuclear membrane has been presented although a great many speculations defining mechanisms for intracellular and intranuclear transport have required a dynamic nuclear membrane. The nuclear membrane and underlying matrix have been implicated as central components of most nuclear events, e.g., replication (Berezney and Coffey, 1976; Shaper et al., 1979), hormone-growth factor stimulation (Vignerie et al., 1978; Yankner and Shooter, 1979), chromatin and nuclear pore assembly (Wunderlich et al., 1976; Giese et al., 1979), viral assembly (Lane and Hoeffler, 1980; Hodge et al., 1977), and carcinogenesis (Clawson et al., 1980). Structurally, the nucleus is a double membrane system with a perinuclear cisturnae between the bilayers. The outer nuclear membrane is studded with polyribosomes and closely resembles the rough endoplasmic reticulum with which it shares continuity. The inner nuclear membrane, on the other hand, has no ribosomes and maintains intimate contact with a cytoskeletal superstructure composed of nuclear matrix proteins termed nuclear lamins (Gerace and Blobel, 1982), chromatin, and RNA. Physical differences have also been reported for the sensitivity of the outer and inner membrane to perturbants (Franke, 1974).

Lateral mobility of nuclear membrane components has been invoked to provide a mechanism by which a two-way communication could be promoted between the inner and outer nuclear membrane, endoplasmic reticulum (Wunderlich et al., 1976; Morré et al., 1979) and other intracellular organelles (Morré et al., 1979). The use of a two-dimensional transport route (Adam and Delbrück, 1968) could greatly facilitate inter-membrane component interactions by means of dimensional catalysis. A two-dimensional diffusive pathway has also been implicated in the macroassembly of annular subunits into nuclear pore complexes in the membrane (Wunderlich et al., 1976), and recent evidence suggests that it serves as a transport mechanism in nucleocytoplasmic transport of RNA and ribosomal precursors (Gonzalez and Kasper, 1981). A summation of possible roles for lateral diffusion of membrane components is presented in Table 1.

Table 1. Role of Two-Dimensional Lateral Transport in Nuclear Activity

1) Communication through intra-cellular membrane "highways"
   a) between organelles
   b) between plasma and nuclear membranes

2) Catalytic enhancement for replicative and transcriptional enzymes

3) Assembly of annular subunits of nuclear pore complex

4) Nucleocytoplasmic transport of RNA and ribosomal precursors

5) Biosynthesis of nuclear membrane proteins

Although the existence of hormone and growth factor receptors in the nucleus is still controversial, their proposed existence may suggest mechanisms of action that could be very similar to plasma membrane dynamics with regard to the need for a dynamic membrane to initiate nuclear activity. The clustering of nuclear receptors may initiate changes in DNA replication or transcription either directly, or by mitigating changes in nuclear matrix interactions, a phenomenon similar to that observed in cell surface events (Schlessinger et al., 1978; Zidovetzki et al., 1981). The direct interplay of cytoskeleton components with membrane receptors has previously been characterized in erythrocytes and the plasma membrane of cells (Koppel et al., 1981; Schindler et al., 1980; Schlessinger et al., 1977). These interactions in the nucleus may ultimately provide: a) the means to signal for specific metabolic and replicative events and b) define, order and maintain specific topological sites and orientations in the nuclear inner membrane for differentiated function. This report describes the diffusion of lectin receptors and lipids in the outer and inner nuclear membrane, and characterizes a method that selectively depletes outer nuclear membrane while retaining the inner membrane.

PREPARATION AND CHARACTERIZATION OF OUTER MEMBRANE DEPLETED NUCLEI

Rat liver nuclei were isolated using modifications (Schindler, 1983) to the method of Kay et al. (1972). Scanning electron micrographs display a relatively smooth membrane surface with ribosomes, pore complexes, and outer membrane blebs (Fig. 1A). To prepare outer membrane depleted nuclei, the nuclei are treated with citraconic anhydride (10-18 mM), a reagent specific for primary amines (Gertler, 1971), and previously employed to alter nuclear DNA and RNA content (Schindler, 1983). Figure 1 is a series of scanning electron micrographs of nuclei treated with 0, 5, and 11 mM citraconic anhydride. A progressive loss of smooth surface material and polyribosomes is observed with a concomitant increase in rough discontinuous areas. At 11 mM reagent there is a mosaic of smooth, rough and vesicular areas. Transmission electron micrographs also show a progressive loss of outer membrane and the emergence of a rather ill-defined rough bumpy surface with occasional glimpses of bilayer (data not shown). Table 1 shows that at 11 and 18 mM reagent concentration, nuclei retain about 8-15% of the DNA, 20-35% of the RNA, and 27% protein. A silver stained SDS polyacrylamide gel demonstrates the polypeptide pattern of citraconylated nuclei (Fig. 2).

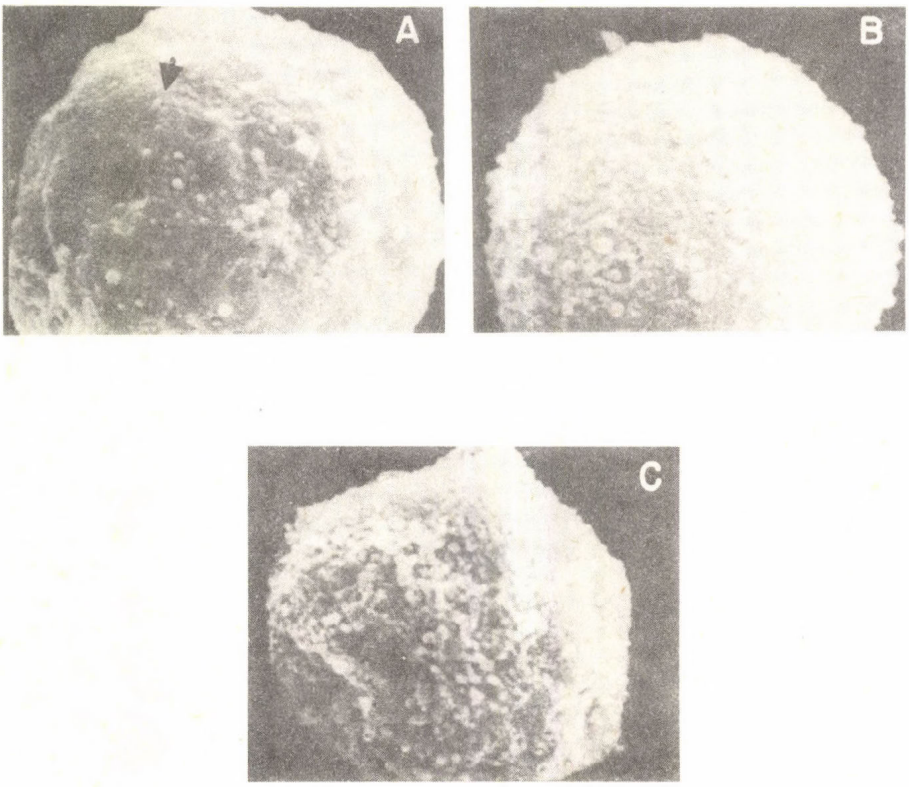

Fig. 1. Scanning and Transmission Electron Micrographs of the Nuclear Periphery Following Citraconylation. Nuclei were isolated and prepared essentially as described by Kay et al. (1972) with minor modifications (Schindler, 1983). Citraconylation was performed as described by Schindler (1983). Scanning micrographs A, B and C are representative of samples modified with 0, 5, and 11 mM citraconic anhydride, respectively. All citraconylation reactions were initiated as follows: 20 $O.D._{260}$ units of nuclei in Buffer B (200 µl) were added to a 1 ml Eppendorf conical tube. The sample was centrifuged for 10 sec at 15,000 g in an Eppendorf centrifuge in the cold (4°C). The supernatant was aspirated and 1 ml of modification buffer (200 mM Hepes 0 1 mM Mg^{++} - 0.25 M sucrose - pH 7.9; the buffer was brought to pH by the addition of approximately 100-120 mM NaOH resulting in 100-120 mM Na^{+}) was added to the pellet. Gentle vortexing was employed to resuspend the pellet. Samples for scanning electron microscopy were fixed in 4% glutaraldehyde for 1 hr, followed by post fixation in 2% OsO_4 for 30 minutes. The nuclei were then added to slides coated with 1% poly-l-lysine (3040 M.W.). Adherance was initiated by allowing a suspension of the prepared nuclei to settle for 5 min on the still damp slide. Samples were gently washed and dehydrated through graded ethanols. These dehydrated samples were then critically point dried, gold coated, and then observed.

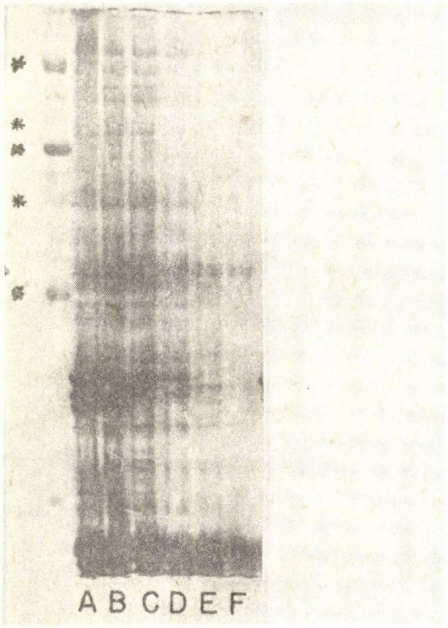

Figure 2. SDS PAGE Gels of Citraconylated Nuclei. SDS PAGE gels (18%) were silver stained for increased sensitivity. Lanes A-F are nuclei treated with 0,1,3,6,12 and 20µl/ml citraconic anhydride. The asterisks are molecular weight standards indicating 200K, 116.2K, 92.5K, 66.2K and 45K respectively.

QUANTITATION OF NUCLEAR MEMBRANE RELEASE FOLLOWING CITRACONYLATION

The loss of nuclear membrane as a function of citraconylation was evaluated using a number of techniques (Fig. 3a). The results using three probes for phospholipid distribution are remarkably similar and suggest that: a) approximately 40-50% of the nuclear phospholipid can be removed by citraconylation, b) that the other 50-60% is relatively refractile to very high modification reagent concentration and high salt, c) that 94-96% of the phospholipid can be released by 1-2% Triton X-100, d) exogenously incorporated phospholipid distributes between the inner and outer membrane, and e) that chemical modification of lipid head group does not play a major role in phospholipid release, since NBD-PE can not react with the reagent.

When 3M urea is employed in conjunction with low levels of citraconic anhydride, a much higher percentage of phospholipid is released from the matrix. This loss of phospholipid is linear (Fig. 3b) and would suggest that the apparent two phospholipid populations have been normalized. The loss of 50-60% of the total phospholipid parallels the disassembly of the nuclear matrix and apparently is most effected by the progressive loss of lamin B, since lamin A and C are effectively solubilized from a precipitable nuclear structure with 3M urea in the absence of citraconic anhydride (Gerace and Blobel, 1982). These results support the conclusions of

Gerace and Blobel (1982) that lamin B may be an inner membrane associated matrix component. The nuclear matrix proteins, lamins A-C, have been demonstrated to be rich in sulphydryl groups that artifactually form disulfide crosslinks following nuclear isolation (Lam and Kasper, 1979; Kaufmann et al., 1983). The possible involvement of these sulphydryl groups in stabilizing inner membrane attachment to nuclear matrix was investigated by reducing whole nuclei with mercaptoethanol (5%), and treating with N-ethylmaleimide (5mM), followed by citraconylation. The data (Fig. 3C) provide evidence that a percentage of phospholipid species that was refractile to high levels of modification is now available for release from matrix. Modification of cytoskeletal SH groups, most significantly spectrin, has been suggested to alter disulfide bond formation between membrane protein SH groups leading to changes in protein-phospholipid interactions in red blood cells (Haest and Deuticke, 1976; Fischer et al., 1978). Such a mechanism may be involved in inner nuclear membrane stabilization. In a related series of investigations, a number of other possible membrane active conditions were employed. Treatment with 1.0-2.5% citric acid has been suggested to release outer membrane (Smith et al., 1969) or approximately half the total phospholipid (Gurr et al., 1963; Virtanen et al., 1977). The results presented in Fig. 3D suggest that only 30% of the [^{3}H] phospholipid is released over a range of concentrations.

Diffusion of Lectins and Phospholipid in the Outer and Inner Nuclear Membrane

Fluorescence redistribution after photobleaching (FRAP) was performed on whole and modified rat liver nuclei to measure the lateral mobility of a fluorescently derivatized phospholipid analog N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidyl ethanolamine (NBD-PE) and a number of fluorescein derivatized lectins, namely wheat germ agglutinin (WGA), concanavalin A (Con A), and succinylated concanavalin A (s Con A). These lectins readily bound to isolated rat liver nuclei while the phospholipid incorporated into the bilayers. Lectin binding appeared to be specific in that it was prevented when the incubation mixture contained the appropriate inhibitory sugar for each lectin (0.2 M α-methylmannoside for Con A and 0.2 M N-acetyl-glucosamine for WGA). Fig. 4 represents a typical bleaching experiment used to determine lipid diffusion. NBD-PE was incorporated into isolated nuclei (Koppel et al., 1981) and single nuclei were then scanned by a galvanometrically controlled moving laser beam focussed to a spot $\leq$ 1 μm. The fluorescence excited by the beam as it traversed the nucleus appeared as a symmetrical double peak profile characteristic of surface labeling on a spherically-shaped structure (Koppel et al., 1980; Sheetz et al., 1980). At a predetermined time, one edge was bleached by an increase in excitation energy ($\sim$5000 X more intense than monitoring intensity) resulting in photobleaching and a destruction of the fluorescence signal at the point of bleaching (Fig 4a, arrow). The entire structure was then repeatedly scanned at monitoring intensities (an intensity at which bleaching is limited). The NBD-PE results in a phospholipid diffusion constant of $3.7 \pm 1.1 \times 10^{-9}$ cm^2/s (Table 2).

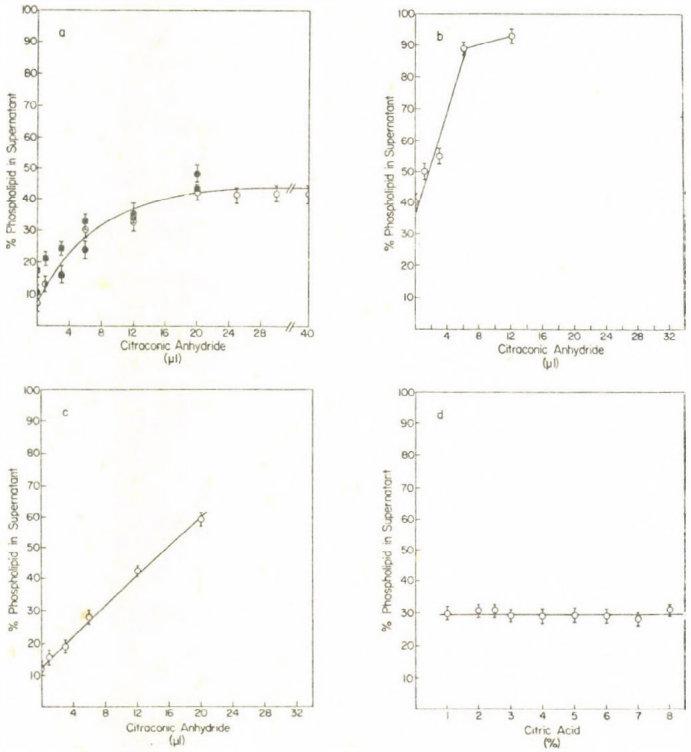

Fig. 3a. Effect of Citraconylation and Membrane Perturbants on Nuclear Phospholipids. Nuclei were citraconylated as described (Schindler, 1983). The initial approach (endogenous) employed the centrifugal separation of the reaction mixture into supernatant and pellet fractions which were extracted with chloroform-methanol (2:1). Phosphate analysis was performed (Ames and Dubin, 1960) and the percent chloroform soluble phosphate in the supernatant was plotted ( ). In another approach (exogenous), radioactive lipid isolated from the membranes of S. typhimuirum G30 (Jones and Osborn, 1977) was solubilized in ethanol and added to whole nuclei. After 15 min of incubation at room temperature and extensive washing, the amount of phospholipid transferred was equivalent to approximately 5-12% of the initial nuclear phospholipid. These nuclei were then treated with modification reagent and analyzed as discussed in the text ( ). A third approach was to incorporate fluorescently labeled NBD-PE into nuclei (exogenous) by addition of NBD-PE in ethanolic solution to nuclei (Koppel et al., 1981) ( ). Following reaction, supernatant and pellet were made 2% in Triton X-100 and the absolute fluorescence was determined in a Perkin-Elmer spectrofluorimeter.

b. Citraconylation was performed in the presence of 3M urea.

c. Nuclei were pretreated with 5 mM β-mercaptoethanol followed by two washes in 50 mM Hepes, 1 mM Mg^{++}, pH 7.5 buffer. These nuclei were then reacted with 10 mM N-ethylmaleimide and analyzed for phospholipid released.

d. Phospholipid alterations are monitored as a function of citric acid concentration (citric acid in water).

The analysis of phospholipid distribution in B, C and D was performed using exogenously incorporated [^{3}H] labeled phospholipids. All phospholipid analyses were corrected to the amount of lamins A-C or lamin B in the case of urea treatment calculated by densitometry of an aliquot of each sample electrophoresed on reduced SDS PAGE gels.

When analyzed as detailed by Koppel et al. (1980) for diffusion on spherical structures, recovery varied between 75-100%. Rebleaching the initial bleach spot resulted in complete recovery with the same calculated diffusion constant. When nuclei are treated with 5 mM citraconic anhydride, lipid diffusion is again observed (Table 2) with the same diffusion constant. Because the lipid distributes itself between the inner and outer membrane (Fig. 3) approximately equally, this diffusion constant apparently reflects mobility, predominantly on the inner membrane. At higher modification reagent concentrations (11 and 18 mM), recovery is still observed (Table 2) suggesting that the segmented lipid areas observed on the nuclear periphery (Fig. 1) maintain continuity with each other. The inner nuclear membrane surface is observed as rough and patchy following the contour of the nuclear matrix (Fig. 1). Such a patchy inner membrane has been observed by others in scanning electron micrographs of outer membrane depleted areas in the nuclei of Xenopus laevis oocytes (Schatten and Thoman, 1978).

The WGA receptors, which are proteins (the nucleus apparently does not contain glycolipids (Franke et al., 1976)), diffuse approximately 10 times slower than the phospholipid. A significant difference is that fluorescence recovery is quite a bit less than phospholipid (40-60%) and would suggest immobile lectin receptors. A Western transfer blot of a PAGE gel of whole nuclear proteins demonstrates a heterogeneous class of proteins capable of binding this lectin (Schindler, M., manuscript in prep.). The data suggests that these mobile receptors are more likely to be found on the outer nuclear membrane (Table 2), since no mobility is observed following major depletion of outer nuclear membrane (at 11 and 18 mM citraconic anhydride). When nuclei are treated with 2% glutaraldehyde for 0.5 h, no recovery is observed for WGA. Fig. 5 presents the fluorescence scans of WGA receptors following various levels of citraconylation. Clearly, the receptors located in the nuclear interior are lost as a function of citraconylation.

The ConA and sConA, on the other hand, demonstrate no diffusions under any circumstances (Table 2). The membrane receptor for this lectin has been localized to a high molecular weight protein suggested to anchor pore complexes to the nuclear matrix (Gerace et al., 1982). The lack of mobility for this protein is consistent with its role as a structural component of the nuclear pore complex or matrix.

Table 2. Diffusion Constants for Phospholipid and WGA Receptors in Nuclear Membrane.

Treatment	% Components/Modified Nuclear Pellet				Diffusion Constant(a)		
mM Citraconic Anhydride	Phospholipid	DNA	RNA	Protein	WGA	Con A	NBD-PE
0	93	100	100	100	$3.9\pm1.3 \times 10^{-10}$	$\leq 10^{-12}$	$3.7\pm1.1 \times 10^{-9}$
5	70	30	60	35	$5.5\pm1.5 \times 10^{-10}$	$\leq 10^{-12}$	$3.8\pm1.3 \times 10^{-9}$
11	67	15	35	27	$\leq 10^{-12}$	$\leq 10^{-12}$	$3.8\pm1.3 \times 10^{-9}$
18	58	8	20	27	---	---	---
2% Triton X-100	5	100	100	--	---	---	---

(a) units of cm^2/s
(b) Mean ± S.E. (generally 10-15 determinations)

The Outer and Inner Nuclear Membrane - Functional Differences

The literature dealing with nuclear membrane separation is fraught with inconsistencies. Although selective solubilization of outer membrane has been suggested to occur following treatment with Triton X-100 (Ben-Porat and Kaplan, 1971), this does not appear to be the case (Aaronson and Blobel, 1974). The use of 2.5% citric acid also appears to be a less than desired agent (Fig. 3D) because of its denaturing effect on nuclear enzymes. Since all work examining the compositional, functional, and structural properties of these membranes rely on one of the aforementioned techniques for membrane separation, the results obtained suggesting major metabolic, enzymatic, and physical differences between them must be most carefully evaluated. Tata et al. (1972) provide controversial evidence that inner nuclear membrane phospholipid is four times more stable to turnover and is found to associate with chromatin. Differences in turnover of phospholipid in both membranes was also reported by Ben-Porat and Kaplan (1962). Conner et al. (1980) demonstrate that approximately 50% of early G nuclear phospholipid is reutilized in membrane reformation during mitosis. A number of investigators find chromosomes carrying inner membrane fragments (Franke, 1974). Gerace and Blobel (1982) present data implicating lamin B, a matrix protein, as a membrane binding protein hooking the cytoskeleton to the membrane. Physically, the inner membrane has been reported to be far more resistant to mechanical stress (Franke, 1974).

The results reported provide support for the view of two membrane systems with very different physical properties. The outer membrane studded with ribosomes is extremely sensitive to chemical modification by a reagent that alters the net charge of lysyl residues by -2 in a manner similar to post-translational modification of amino acids by protein phosphorylation. During modification, the outer membrane vesiculates and its physical continuity with the nucleus is broken. The inner nuclear

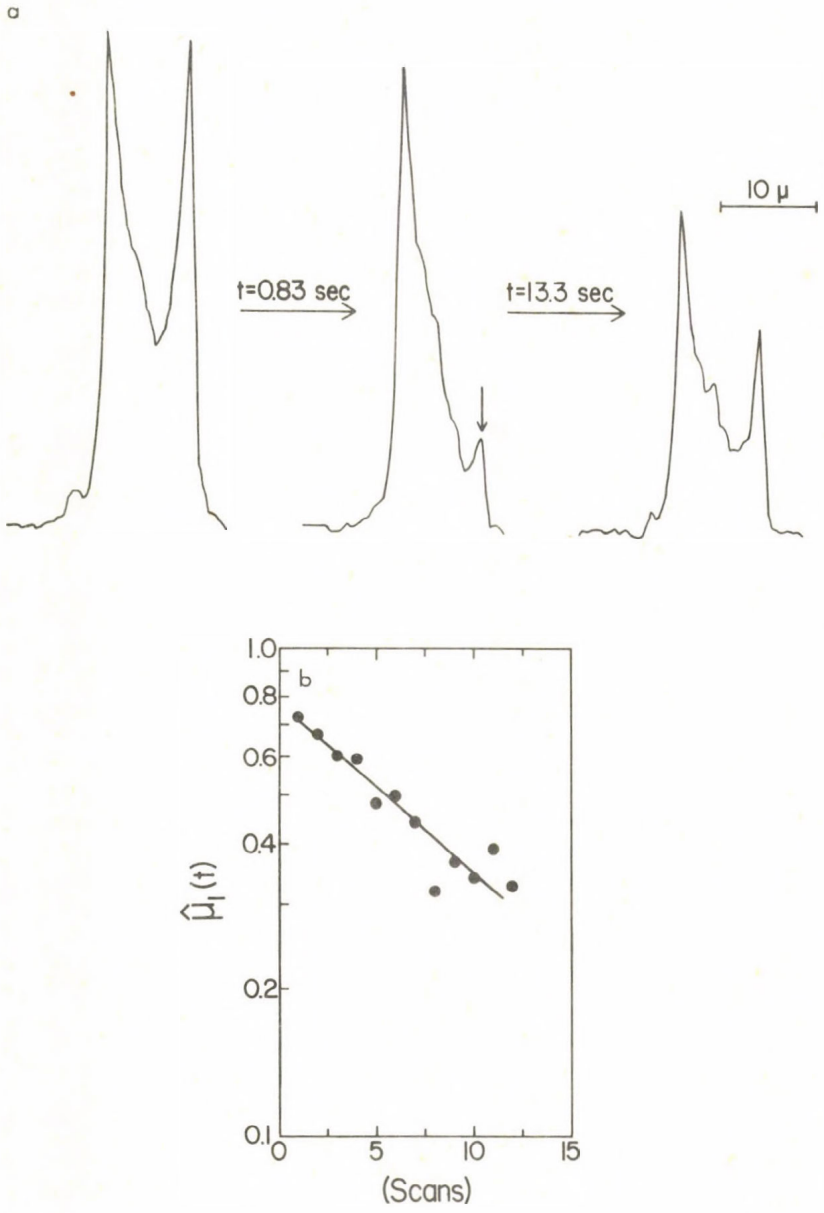

Figure 4. Fluorescence Redistribution of NBD-PE in Nuclear Membranes After Photobleaching. N-4-nitrobenzo-2-oxa-1,3-diaxole-derivatized phosphatidylethanolamine (NBD-PE) purchased from Avanti (Birmingham, AL)

was used as a probe for phospholipid mobility. Incorporation of this labeled lipid into modified and unmodified nuclei was done in the following manner: NBD-PE in ethanol (3.75 µg/3 µl) was added to 20 $O.D._{260}$ units of nuclei (35) in 1 ml of labeling buffer (50 mM Hepes - 1 mM Mg^{++} - pH 7.5). The suspension was incubated with shaking for 20 min at 25°C. The nuclei were then pelleted in an Eppendorf centrifuge for 3 sec at 15,000 g (25°C). Supernatant was removed and fresh labeling buffer was added (1 ml) followed by resuspension of the nuclei with a glass rod. This washing procedure was performed 3 times. Fluorescein labeled wheat germ agglutinin (WGA), concanavalin A (conA) and succinylated concanavalin A (s ConA) (Vector Labs, Burlingame, CA) were used to measure glycoprotein mobility. Fluorescein labeled lectins (10-30 µg/ml) were added to 20 $O.D._{260}$ units nuclei suspended in 1 ml labeling buffer. The reaction was allowed to proceed at 25°C for 10 min. The washing procedure employed was the same as that used for NBD-PE labeling. Ten µl of the labeled nuclear suspension were placed on a slide and a coverslip was applied. The coverslip edges were then sealed with wax.

The data obtained from the FRAP method consists of a series of discrete points composing a fluorescence excitation scan produced by the controlled movement of a focussed laser beam across the nucleus. The redistribution of labeled molecules after a localized photobleaching pulse was analyzed with a normal-mode analysis, following the approach of Koppel et al. (1980). NBD-PE and fluorescein labeled lectin fluorescence was monitored with an incident wavelength of 4,765A and a combination of Leitz dichroic mirror TK510 and barrier filter K510.

b. Quantitative Analysis of Recovery for NBD-PE in the Membranes of Whole Rat Liver Nuclei. μ_1 (t) is the experimental estimate of the normalized first moment of the fluorophore concentration distribution. Each scan is equivalent to 0.85 s.

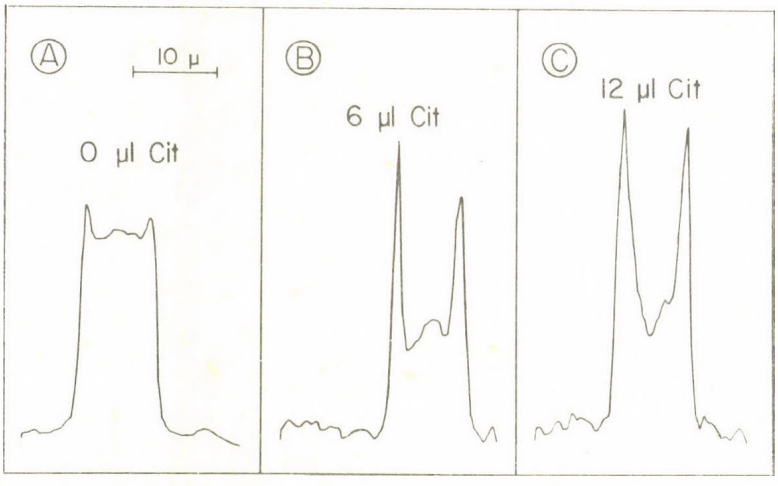

Figure 5. Fluorescence Profile of Fluorescein-WGA Binding to Untreated Whole Rat Liver Nuclei (a), treatment with 6 µl/ml (5.5 mM) citraconic anhydride (b), and treatment with 12 µl/ml (11 mM) citraconic anhydride (c). The laser excitation scans are performed by a beam focussed to a 1-2µ diameter. The scans were all recorded on the same scale.

membrane, on the other hand, maintains a tight association with the submembranous array of lamins and pore complexes. These attachments appear not to be greatly affected by the removal of large amounts of lamins A and C, but are abrogated with the progressive loss of lamin B following citraconylation in the presence of urea. The possibility of lamin B providing a protein connection for the membrane to nuclear matrix in a manner reminiscent of ankyrin in red blood cells has been suggested (Gerace and Blobel, 1982) and is consistent with our results. The sensitivity of inner membrane release to sulphydryl reagents may suggest that the lamins stabilize the membrane by the formation of disulphide linkages with inner membrane integral membrane proteins. The different sensitivities for membrane perturbants may have specific mechanistic implications for the particular role of each membrane in the biosynthetic repertoire of the cell. The outer nuclear membrane has been suggested to be a source of membrane material for the construction of intracellular organelle membranes (Morre et al., 1979), because it continuously produces blebs which are capable of fusing to form extended membrane structures. The high phosphatidyl choline content (60% total phospholipid vs. 30% in plasma membranes), low cholesterol (5-10 times less than plasma membrane) coupled with absence of highly charged neuraminic acid containing glycoproteins provide the physicochemical requirements for a very "fluid" and fusogenic membrane system enhancing the likelihood of vesicle transfer events between the outer nulcear membrane and other intracellular membranes. The inner nuclear membrane, however, is resistant to release by chemical modification at reagent levels capable of removing membrane from red blood cells (Steck and Yu, 1973), and remains firmly attached to the submembraneous lamin assembly. Possible biological reasons for this type of tight association may involve the nature of biochemical events suggested to occur at the matrix level. The origin of replication has been suggested to be attached to the inner membrane-matrix not only in nuclei, but in analogous membranes in bacteria, mitochondria, and chloroplasts. The existence of a two-dimensional catalytic surface for the organization of replicating enzymes and the maintenance of a specific microenvironment could require a stable membrane-matrix structure. In addition, the hydrophobic environment existing at the matrix-membrane interface may be extremely important in maintaining a specific folding pattern of matrix associated DNA that can be quickly altered following membrane breakdown at the onset of mitosis. Finally, the membrane has been observed to stay attached to condensed chromatin following nuclear disassembly (Franke, 1974). This membrane may provide additional recognition information for correct nuclear reassembly. Such reassembly of membrane enclosed chromosomes (karyomeres) by subsequent fusion has been observed (Gall, 1964).

Intracellular Membrane Mobility - Diffusion Highways

The ability of plasma membrane components to laterally and rotationally diffuse with subsequent redistribution in the plane of the membrane has been a central element in present theories on transmembrane signaling, endocytosis, virus assembly, and the development of specific cell surface distributions of membrane bound molecules (Axelrod et al., 1978; Cherry, 1979; Reidler et al., 1981; McClain and Edelman, 1979). Although a great deal of evidence has been generated to support the role of lateral mobility in the plasma membrane as a vehicle for signal transduction in the cell's chemical dialogue with its environment, no body of data is available with regard to the role of lateral transport in the intracellular movement of proteins, lipids, and glycolipids. This process may be of

paramount importance in the vectorial movement of membrane components between adjacent compartments under conditions in which the membranes are contiguous (Morré et al., 1979). Such structural continuity exists between outer nuclear membrane and rough endoplasmic reticulum, between rough and smooth endoplasmic reticulum, between Golgi and secretory vesicles, and ultimately between secretory vesicles and plasma membranes (Morré et al., 1979). Additional membrane continuities have connected endoplasmic reticulum with secretory vesicles, endoplasmic reticulum, Golgi, and lysosomes, endoplasmic reticulum and outer mitochondrial membranes, and microbodies and endoplasmic reticulum (Morré et al., 1979). Given these structural continuities, it may be appropriate to visualize the internal membrane systems as providing two-dimensional transport surfaces or highways that have particular exits, preferred routes, detours and shuttle mechanisms (intracellular vesicles) that allow the vectoral transport and subsequent intermixing of intracellular organelle and plasma membrane components destined for export or redistribution. The advantages of such a system when compared with three-dimensional diffusion mechanisms are numerous and have been elegantly detailed in the work of Adam and Delbrück (1968).

The results of the present research support the possibility that lateral mobility offers a means of transport for intracellular communication. The outer nuclear membrane is dynamic with proteins and lipids able to undergo long range lateral transport. The continuity between outer nuclear membrane and endoplasmic reticulum suggest that the phospholipids are capable of movement between the two membranes. This is reflected in their similar phospholipid composition. Since unhindered diffusion of membrane components through the intracellular compartments would result in similar phospholipid composition, the reciprocal relationship for phosphatidylcholine content with that of phosphatidylethanolamine and cholesterol observed when comparing nuclear to plasma membrane compartment suggests the likelihood of vesicles mediating the movement of membrane components between bounded intracellular membrane systems resulting in organelle phospholipid compositional specificity without phospholipid intermixing occurring due to diffusion. Within the nucleus itself, however, there would appear to be dynamic continuity for phospholipid movement between outer and inner nuclear membrane past the annular subunits comprising the pore complex, suggested by the rapid equilibration of exogenously incorporated phospholipid (<1 min, data not shown). This appears to provide a mechanism for mobilizing phospholipid from outer membrane to replace inner membrane phospholipid and perhaps a pathway for phospholipid deposition on nuclear matrix as a function of nuclear contraction and expansion. Wheat germ agglutinin receptors, on the other hand, appear to have mobile species on the outer nuclear membrane but demonstrate no mobility on the inner membrane. These results in conjunction with a report on the immobility of a viral protein (Puddington et al., 1983) most probably located on the inner nuclear membrane may suggest that inner membrane proteins are maintained in a specific topological localization by matrix proteins or submembraneous DNA-RNA interactions with integral membrane proteins. Evidence derived by viral assembly studies suggest that the inner nuclear membrane may be a static mosaic of functional domains (Ben-Porat and Kaplan, 1971). On the other hand, the mobility of proteins in the outer membrane further supports the idea that the endoplasmic reticulum may serve as a diffusion highway to redistribute glycoproteins throughout the intramembrane system. Specific interactions with intramembraneous non-mobile protein anchors (Schindler et al., 1980) or submembraneous

structures (Koppel et al., 1981) may serve to act as diffusional gates catching proteins in defined areas to establish a topologically specific membrane region. The two nuclear membranes may thus serve two very different functions. The outer membrane provides: (a) an intracellular communication pathway, (b) a biosynthetic source of internal membranes, and (c) a source of phospholipid for inner nuclear membrane. The inner membrane, on the other hand, offers: (a) a two-dimensional catalytic surface for replicating enzymes and DNA attachment, (b) a microenvironment for specific enzymatic events and DNA/RNA packaging, and (c) domains for viral assembly.

Acknowledgements

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ERNSTER:

Are the inner and outer membranes continuous (in terms of phospholipid bilayer) at the level of the pores?
Does one know anything about the contents of the space between the two membranes?
It seems to be well established there exists a cytochrome P450-linked monooxygenase system associated with the nuclear membrane(s). Does one know which of the two membranes is the site of this system?

SCHINDLER:

Yes, there is good TEM evidence for this.
There has been no good experimental approach to this problem.
There is some microscopic (TEM) evidence that the P450 is on the outer membrane. But again, this is not entirely conclusive.

SZABÓ:

Can you exclude the possibility that you introduce artificial cross-links with the preparation procedure?

SCHINDLER:

Yes, using reducing conditions during bleaching. In addition, our modification reagent is incapable to X-link the proteins.

BHARGAVA:

There is an interesting recent evidence supporting one of the functions you have ascribed to the inner nuclear membrane. Prem Veer Reddy and Arthur Pardee of Boston have shown that conversion of cytidine to dCTP, as well as perhaps many other reactions of the DNA synthetic pathway (including the DNA polymerase-catalysed replication) occur in a nuclear membrane-associated multienzyme complex. This complex would not accept, for example, preformed dCTP.

ZS.-NAGY:

You probably know that the nucleus has a higher water content (about +2-3 % by weight) as compared to the cytoplasm. The structure of proteins you showed may play an important role in the maintenance of this difference.

DAMJANOVICH:

The different conformations of the chromatin or the DNA in the chromatin like Z-DNA might influence the chromatin-membrane interactions. What is your opinion about the possible influence of these events upon the mobility and function of nuclear membrane elements?

SCHINDLER:

Altered chromatin at the membrane-matrix interface may serve to control the topological arrangement and movement of inner membrane intramembranous proteins in a manner analogous to spectrin-band 3 interactions in the red blood cell.

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CELL SURFACE DYNAMICS AND DISTANCE RELATIONSHIP OF INTEGRAL MEMBRANE PROTEINS

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The cytoplasmic membrane, the cell surface can be regarded as a mechanical barrier isolating the interior of the cell from the environment. The cell machinery is, however, completely dependent on a dynamic interaction between the outside and the inside of the cell. This interaction involves the interchange of signals, chemicals and nutrients between the environment and the interior of the cell. Cells are capable to respond very specifically to certain important signals. It is customary to distinguish early and later events in some specific cellular response both containing numerous elementary steps.

Among these elementary steps association phenomena, redistribution of receptors or other membrane constituents occur frequently inside the cytoplasmic membrane, the very place of the signal transmission. Proteins, hormones and various ligands having bound to their respective receptors might trigger local aggregation, cause the formation of molecular products, activate various transport systems. The change in the intracellular concentration of certain ions and nutrients could in turn activate intracellular biochemical processes, stimulate protein and nucleic acid synthesis.

It was shown that the low density lipoprotein binds to clustered LDL-receptors on defined areas of human fibroblasts (Anderson et al., 1977). This aggregation is a ligand independent phenomenon. On the other hand, the binding of peptide hormones such as insulin (Kahn et al., 1978), epidermal growth

factor (Schlessinger et al., 1978), occurs to the dispersely distributed receptors. The unoccupied homogenously distributed receptors undergo local aggregation upon binding the hormone. These microaggregated hormone receptor complexes cluster at coated regions of the membrane. The immobile clusters then interact with other sub- and/or intramembrane assemblies leading to the internalization and degradation of the hormone. Mentioning just a couple of other examples we refer to the ligand induced aggregation of α_2-macroglobulin (Maxfield et al., 1978) receptors, nerve growth factor receptors (Levi et al., 1980) lymphocyte surface immunoglobulins (Taylor et al., 1971), lectin receptors, H-2 (Henis and Gutman, 1983) and HLA antigens (Petty et al., 1980).

The elucidation of the structure and function or the detailed mechanism of a complex response of a cell to specific triggering signals is a widely studied problem in molecular and cell biology. Lateral and rotational motions of membrane components are often of central importance in allowing certain interactions between different constituents of the cytoplasmic membrane and/or other cell components. On the other hand, changes in the interactions during a specific response of the cell may reflect in altered rotational or lateral mobility of selected membrane proteins.

Boyse and others observed a supramolecular patterning on mouse thymocytes (Boyse et al., 1968; Flaherty and Zimmerman, 1979). They found that certain alloantigens were in close proximity. A conclusion can be drawn that this more or less defined steric arrangement of specific antigens may have importance in immunological recognition.

The appearance of the fluid mosaic membrane model (Singer and Nicolson, 1972) has swept away the significance of the results by Boyse et al. However, there is a still growing amount of evidence that the extent of the mobility of the intramembrane proteins and glycoproteins is rather limited. The almost general finding that the lateral mobility of these membrane constituents is 2-3 orders of magnitude lower compared to those of the same proteins in reconstituted membranes underlines the relevance of the blocking test resulted supramolecular

pattern and its theoretical implications. We also put forward that the receptor pattern can contain specific information, which may have a role in recognition processes and in the cell to cell communication (Damjanovich et al., 1981).

Owing to these facts, and, since aggregation and association phenomena can be investigated by studying topological relationships, the techniques allowing distance measurements on the cell surface seem to be as important as the mobility measurements.

Methods for the determination of rotational mobility

Information on these interactions manifesting in rotational mobility changes has been obtained by using a variety of depolarization techniques.

Rotational correlation times of intramembrane proteins or glycoproteins undergoing rotational Brown motion fall usually into the microsecond-millisecond time range. Depolarization techniques allowing to study these slow processes have been developed involving a spectroscopic state with a life-time comparable or longer than the correlation times in question. In addition to the absorption and emission measurements from triplet states and measurements of the delayed fluorescence the depolarization of the fluorescence depletion enables us to follow slow rotational diffusion. The latter method developed recently outstands with its higher sensitivity.

The basis of all spectroscopic methods measuring rotational parameters of macromolecules is the photoselection of an oriented subpopulation from an initial ensemble of macromolecules having randomly oriented absorption transition dipoles. The ordered character of the photoselected subpopulation follows from the anisotropic nature of the absorption, whereby the probability of the selection (absorption) is proportional to $\cos^2 \phi$, where ϕ is the angle between the absorption dipole of the molecule and the electric vector of the incident plane polarized light. Signals arising from an ordered subpopulation reflect the extent of nonrandomness of the photoselected ensemble as anisotropic emission (in the case of fluorescence, phosphorescence or delayed fluorescence anisotropy decay measurements) or dichroic absorption (in the case of transient triplet-triplet absorption technique).

The measured anisotropy parameter is defined either as the absorption anisotropy:

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \qquad (1)$$

or the emission anisotropy:

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \qquad (2)$$

The anisotropy is usually time dependent after the photoselection (t=0) because of different randomizing processes. Here $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbances at time t for the light polarized parallel and perpendicular with respect to the polarization of the excitation light. $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the fluorescence intensities at time t detected through a polarizer with transmission direction parallel or perpendicular to the polarization of the excitation light.

The absorption or emission components of the r(t) anisotropy parameter display a time dependence because of the decay of the spectroscopic state involved in the photoselection, because of energy transfer processes (transferring the excitation energy from initially selected chromophores to nonselected ones) and because of the randomizing effect of the rotational Brown motion. The r(t) was shown not to depend on the decay of the mentioned spectroscopic state in the case of the phosphorescence and delayed fluorescence depolarization methods or the transient dichroism (triplet-triplet absorption) measurements. All the remaining depolarization processes result in an exponential decay of the anisotropy of the form

$$r(t) = r_{\infty} + (r_0 - r_{\infty}) \sum_{i=1}^{m} a_i \, e^{-\frac{t}{\theta_i}} \qquad (3)$$

The number of the exponential terms, m, depends on the particular type of the rotor. In cases where the range of rotations is limited the randomization ends up in a finite anisotropy value r_{∞} at $t \to \infty$. r_0 denotes the initial value of the aniso-

tropy at t=0, n_i (i=1,2,...n) are rotational correlation times with amplitudes a_i.

Method for the determination of lateral mobility

Lateral mobility of fluorescently labeled membrane components can be measured with the fluorescence recovery after photobleaching (FRAP) technique. In this method an intensive laser beam is focused to a small area of 1-5 μm diameter of a cell through a fluorescence microscope. Fluorophors on this region can be irreversibly bleached by a short exposure to this intensive light. After the bleaching pulse a 10^3-10^4 times attenuated monitor beam allows to follow the recovery of the fluorescence from the same spot of the cell surface indicating the diffusion of nonbleached labeled components from the surface outside of the bleached area. The analysis of the kinetics of the process yields the diffusion constant, while that of the steady-state fluorescence levels allows to calculate the mobile fraction of the investigated entity.

Method for the determination of spatial relationships

To measure distance relationships between specific sites on a cell surface the most applicable method is the measurement of fluorescence energy transfer. This technique has been applied in studies on mammalian cells using cell suspension with steady-state fluorimeter, or on a single cell basis with microscope or flow cytometer. Up till now all of these measurements have yielded either population averaged energy transfer data, or relative energy transfer efficiencies.

Recently we have developed a flow cytometric method for the determination of the absolute value of resonance energy transfer efficiency on single cells (Trón et al., 1983).

In a system containing both donors and acceptors (like a cell labeled with both donor and acceptor conjugated ligands) several parameters have to be known to calculate the energy transfer efficiency. The first of them is a fluorescence intensity containing the intensity due to the transfer excited at the donor absorption band and detected in the acceptor emission band. However, this intensity has to be corrected for the directly excited fluorescence of the acceptor, a

finite contribution to the former. Finally the correlated (true energy transfer) signal has to be normalized to the number of donor excitations. With other words, besides the necessary spectroscopic features of the donor and acceptor three entities have to be measured for each single cell. This is a hard problem in the case of most of the donor-acceptor pairs, so we have decided to detect three independent fluorescence intensities (I_1, I_2 and I_3) whose interrelationships with the transfer efficiency can be expressed analitically.

These expressions and the relations for the fluorescein-rhodamine donor-acceptor pair are as follows:

$$I_1(488 \rightarrow 535) = I_F(1-E) \tag{4}$$

$$I_2(488 \rightarrow >590) = I_F(1-E)S_1 + I_R S_2 + I_F\, E\, \alpha \tag{5}$$

$$I_3(514 \rightarrow >590) = I_F(1-E)S_3 + I_R + \frac{S_3}{S_1} I_F\, E\, \alpha \tag{6}$$

I_1, I_2 and I_3 are three fluorescence intensities belonging to a particular double labeled cell with excitation and detection wavelengths indicated in the parentheses measured with a dual wavelength excitation laser and an appropriate double phototube detection system (Fig. 1). E is the energy transfer efficiency; S_1, S_2 and S_3 are corrections for the spectral overlap associated with direct excitation of the respective fluorophors:

$S_1 = I_2/I_1$ determined using cells labeled only with the donor

$S_2 = I_2/I_3$ determined using cells labeled only with the acceptor

$S_3 = I_3/I_1$ determined using cells labeled only with the donor.

I_F and I_R denote the theoretical unperturbed fluorescence intensities in the absence of energy transfer for fluorescein (I_1) and rhodamine (I_3), respectively.

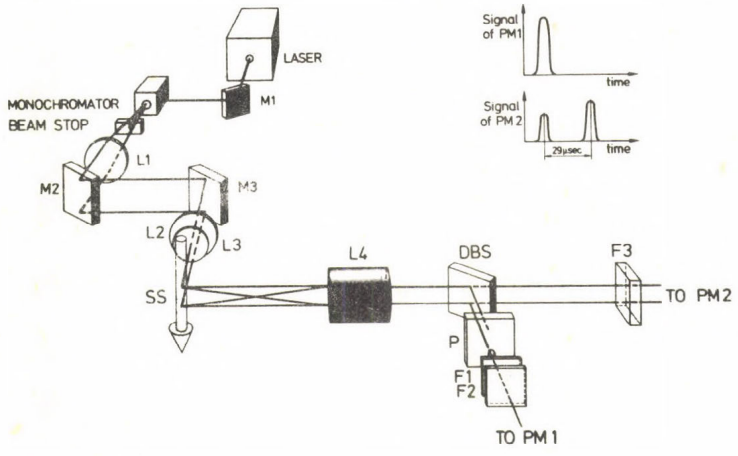

Fig. 1: The 488 nm and 514 nm lines of a 15 W argon ion laser working in "all line" mode were selected and focused to the sample stream, SS, with the aid of mirrors (M) and lenses (L_1-L_3). The cells carried by SS intersected the 488 nm and 514 nm lines at different heights so that the excitations at these wavelengths were separated both spatially (by 0.1 mm) and temporarily (by 29 μsec). Using a dichroic beam splitter (DBS) a P pinhole and appropriate filters (F_1-F_3). PM 1 detected the fluorescence intensity in the fluorescein emission band and PM 2 that in the rhodamine emission band.

The missing intensity from the fluorescence of the fluorescein, because of the loss due to the energy transfer will appear as a surplus in the intensity of rhodamine emission. The detectibility of an excited fluorescein molecule, however, deviates from that of an excited rhodamine molecule. Factor α compensates for this difference.

The solutions of the set of equations (4)-(6) for $A = \frac{E}{1-E}$ and E are

$$\frac{E}{1-E} = \frac{1}{\alpha} \left\{ \frac{(I_2 - S_2 I_3)}{\left(1 - \frac{S_3}{S_1} S_2\right) I_1} - S_1 \right\} \qquad (7)$$

$$E = A/(1+A) \qquad (8)$$

The value of α can be determined from the detected mean relative fluorescence intensities of single labeled cells incubated with saturating concentrations of either donor or acceptor conjugated ligands. The ratio of these signals has to be corrected for the different number of binding sites, fluorophor/ligand labeling ratios of donor and acceptor conjugated ligands and the different absorption coefficients of donor and acceptor at the excitation wavelength.

The excellent statistics provided by the flow cytometric methods allow reliable transfer efficiency determinations and enable us to detect subpopulations of a sample heterogeneous regarding the transfer efficiency. We have worked out a procedure, too, based on an error propagation analysis to estimate the biological variability of the individual cells with respect to E. This parameter can be used as an additional one to characterize a given population.

Results and Discussion

Here we report on energy transfer, rotational and lateral mobility measurements carried out on T41 LDBH(C3HxDBA/2) F_1 cells (Holtkamp et al., 1981). For these investigations we have used fluorescein-isothiocyanate (FITC) and tetramethylrhodamine-isothiocynate (TRITC) conjugated concanavalin A (F-Con A and R-Con A) as well as FITC and TRITC conjugated monoclonal anti-H-$2K^k$ antibodies coded H-100 27/55 and H-100 30/6 (Lemke et al., 1979). Details of sample preparation are given in the legends to the figures.

The proximity of the Con A binding sites was investigated on T41 cells using a computer controlled flow cytometer

equipped with a dual wavelength excitation input optics (Fig. 1). The frequency distributions of the energy transfer efficiency were calculated from the stored I_1, I_2 and I_3 data measured for each individual cell according to equations (7)-(8). Numerical values of S_1, S_2, S_3 and α were determined according to Trón et al. (1983) using single labeled samples.

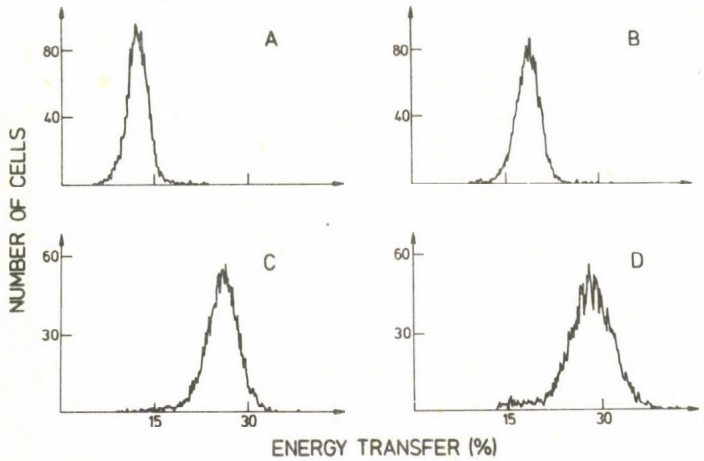

Fig. 2: Frequency distribution of energy transfer efficiency of T41 cells labeled both with F-Con A and R-Con A. TRITC/FITC ratio of labeled Con A bound to the cells: 0.3 (A), 0.6 (B), 1.2 (C) and 2.3 (D). Cells were grown in Dulbecco's minimum essential medium containing 10 % fetal calf serum and antibiotics. Before use they were washed twice with cold phosphate buffered saline and incubated (at $2x10^7$ cell/ml) with 200 µg/ml total Con A concentration at 0^oC for 60 minutes. After this step cells were washed and fixed in 1 % formaldehyde.

The distribution curves were shifted to higher E values as we increased the acceptor to donor ratio keeping the total Con A concentration at saturation level (Fig. 2). From the mean values of these distributions the average density of the Con A binding sites were calculated: $1.1x10^4$ μm^2 (Trón et al.

1983). Assuming an even distribution on the cell surface (with a diameter of 15 μm) the average density calculated from the total number of Con A binding sites was 6.2×10^3 μm^2. The deviation of this value from the one calculated from the energy transfer efficiency distribution can be interpreted as the consequence of a non-random distribution of the Con A binding sites. Instead of a disperse distribution the binding sites are forming submicroscopic microaggregates.

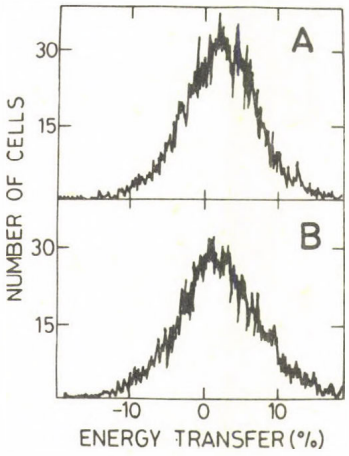

Fig. 3.
Frequency distribution of energy transfer efficiency between A./ FITC-H-100 27/55 and TRITC-H-100 27/55; B./ FITC-H-100 30/6 and TRITC-H-100 30/6 monoclonal anti-H-$2K^k$ antibodies bound to T41 cells. TRITC/FITC ratios were 2. During the incubation the total antibody concentration was kept at saturation. Other details are the same as described in legend to Fig. 1.

Similar measurements on T41 cells labeled with FITC and TRITC conjugated anti-H-$2K^k$ antibodies competing for the same antigen determinants of the antigen (Szöllősi et al., 1982) resulted in an E distribution with average values very close to zero (Fig. 3). This fact argues against the clustering of the H-$2K^k$ antigens.

Energy transfer measurements were carried out between donor labeled H-$2K^k$ antigens and acceptor labeled Con A binding sites (Fig. 4). All the detected distributions measured with samples of different acceptor concentrations can be characterized with mean values significantly higher than zero. The average energy transfer efficiency using saturating amount of R-Con A was very close to the highest E value measured for the F-Con A R-Con A transfer. This fact indicates an interaction between the H-$2K^k$ antigens and Con A receptors. Both

membrane components can be capped applying a second antibody against the anti-H-2K^k antibody or incubating the cells with Con A at a concentration between 5-20 µg/ml at 37°C. If both H-2K^k and Con A receptors are capped simultaneously they move to the same pole of the cells. However, this interaction cannot mean an association or complex of the two membrane components as any of them can be capped without altering the disperse distribution of the other one.

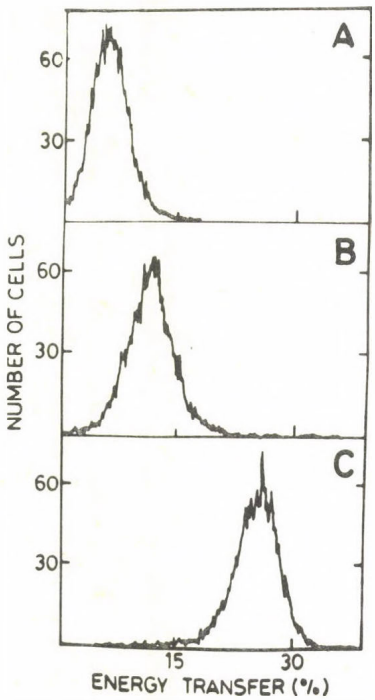

Fig. 4.
Frequency distribution of energy transfer efficiencies between FITC conjugated monoclonal H-100 27/55 anti-H-2K^k antibody and R-Con A bound to T41 cells. During the labeling at 0°C for 60 minutes the total lectin concentration was kept at saturation level while the concentration of R-ConA was A./ 12 µg/ml; B./ 30 µg/ml; C./ 200 µg/ml respectively. Other details are the same as described in the legends to Figs. 1. and 2.

Lateral diffusional mobility of H-2K^k antigen was measured using the FRAP technique (Damjanovich et al., 1983). Cells were labeled either with FITC or TRITC conjugated anti-H-2K^k antibodies. The diffusion coefficients were calculated according to Axelrod et al. (1976). The measured value of the apparent lateral diffusion coefficient was $5 \pm 3 \times 10^{-10}$ cm/s^{-1}. This value was actually the same measured either with whole antibodies H-100 27/55 and H-100 30/6 or with F_{ab} fragments of H-100 27/55 monoclonal antibody in the temperature range of

10-37°C. The lateral motion of the H-2K^k antigen is highly restricted as the measured mobility is about 2 orders of magnitude lower than that of proteins of comparable size in artificial membranes.

The restriction is probably not because of the aggregation of the H-2K^k antigens as it was ruled out by the energy transfer experiments. To check whether it can be connected with the association of the antigens with other membrane components yielding high molecular weight complexes we have carried out rotational mobility measurements using the phosphorescence depolarization method. Experimental data were fitted to a single exponential decay (eq. 3 with m=1).

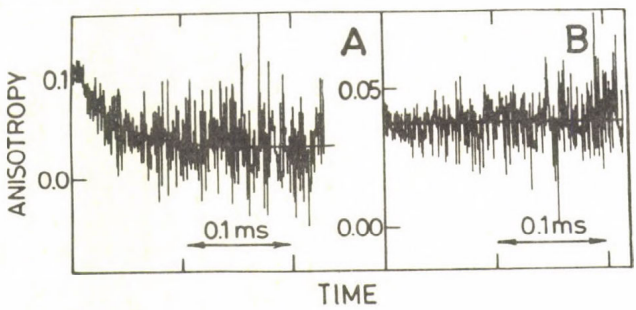

Fig. 5: Phosphorescence anisotropy kinetics of antibody-H-2K^k complexes. The smooth lines represent the results of the computer fitting of the experimental curves. T41 cells were labeled with A./ eosin conjugated F_{ab} fragments of H-100 27/55 anti H-2K^k antibody; b./ eosin conjugated H-100 27/55 anti-H-2K^k antibody followed by an incubation with polyclonal rabbit anti-mouse immunoglobulin. Phosphorescence measurements were carried out at 25°C.

A representative anisotropy decay curve is displayed on Fig. 5.A for a sample of T41 cells labeled with eosin conjugated F_{ab} fragments of H-100 27/55 anti-H-2K^k antibody. The rotational correlation time was 22 μsec and it did not change

significantly upon varying the temperature between 37°C and 4°C, or using eosin conjugated whole antibody instead of the F_{ab} fragments. Very similar time course was found when the cells were incubated prior to the eosin conjugated H-100 27/55 labeling with unlabeled Con A or H-100 30/6, another monoclonal anti-H-$2K^k$ antibody not competing with H-100 27/55 (Damjanovich et al., 1983). The antigen-antibody complexes became totally immobile on the time scale of the experiment when the eosin conjugated anti-H-$2K^k$ antibody was capped with a second antibody (Fig. 5.B).

The results of these measurements are consistent with uniaxially freely rotating species of molecular weight of about 60 000 arguing against H-$2K^k$ aggregates or other high molecular weight complexes of H-$2K^k$ antigen and other intramembrane proteins or glycoproteins.

In conclusion: from distance measurements based on fluorescence energy transfer and the measurements of rotational and lateral mobilities we got evidence that Con A receptors are aggregated on the surface of T41 cells. In contrast, H-$2K^k$ antigens seem to be dispersely distributed and freely rotating but restricted in lateral diffusion. This restriction cannot be due to high molecular weight complexes of H-$2K^k$ antigens and other membrane constituents, but it probably reflects a direct or indirect interaction with submembraneous structures.

Finally we report preliminary data of lateral mobility measurements on normal and Rauscher virus infected C3H/He mouse spleen cells (Fig. 6). Lateral diffusion constants of the H-$2K^k$ antigen-H-100 27/55 antibody complex were measured for normal and transformed cells ($4\text{-}5\times10^{-10}$ cm^2/s) and compared to that of a xenotropic virus related antigen XenCSA (Morse et al., 1979) of virus infected cells in complex with fluorescein labeled anti-XenCSA $F(ab)'_2$ antibody fragments. The lateral mobility of the latter complex by and large agrees with typical values published for membrane proteins of similar molecular weight. These measurements, to be completed with rotational diffusion and distance measurements in the future, may yield information regarding the interaction between the major histocompatibility antigen complex and viral antigens.

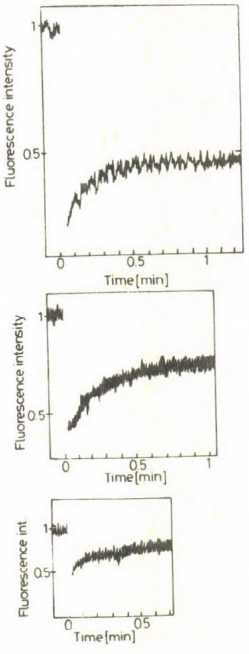

Fig. 6.
Fluorescence photobleaching recovery curves of FITC conjugated antibody-antigen complexes of normal (A) and Rauscher virus infected (B and C) C3H/He mouse spleen cells. Cells were labeled with FITC conjugated H-100 27/55 anti-H-$2K^k$ antibody (A and B) or FITC conjugated anti-XenCSA $F(ab)'_2$ fragment (C). Preparation of the samples was similar to that described in legends to Figs. 2 and 3.

Similar investigations may give a contribution to a better understanding of cell mediated cytotoxicity and cellular recognition processes.

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DISCUSSION

RESCH:

Your experiments concerning the transfer between Con-A receptors were done with native cells and tetravalent Con-A. As under these conditions Con-A induces receptor aggregation, you can make only estimations about receptor distances in this disturbed, i.e., aggregated state. However, statements about receptor clustering in native cells are not possible.

TRÓN:

When energy transfer has been used for studying Con-A receptor distances the lymphoma cells were kept at $0^{o}C$ during the labeling procedure. After a 60 min incubation of the cells with mixture of FITC-Con-A and TRITC-Con-A the samples were washed twice or three times with ice cold PBS and fixed with cold paraformaldehyde. These conditions and the applied high total lectin concentration ruled out heavy patching and capping. Cells labeled according to our protocol, displayed an even "ring type" fluorescence pattern under the microscope. The aggregation concluded from the transfer efficiency measurements is submicroscopic. From our data presented we cannot decide whether this microaggregation is a preexisting association of the receptors or a "disturbed state" evoked by the binding of the tetravalent lectin. To clear out these points we are planning to make similar experiments using either succinylated Con-A or prefixed cells.

BHARGAVA:

What is the maximum distance over which the energy transfer, you mentioned, can take place?

Is there any known case of two fundamentally different entities on the cell membrane being spatially linked so that they can move only together?

Would you comment a little more on the "co-capping" of the Con-A receptors and the H-2 antigen?

TRÓN:

It is widely accepted that distance measurements based on Förster-type energy transfer can be carried out in the range of 2-10 nm. Cantor and Schimmel (Biophysical Chemistry Part II, p. 449; Freeman and Company, San Francisco, 1980) quote a distance of 5 nm. The actual range depends on the critical distance, R_0 which is determined by the spectral overlap between donor emission and acceptor absorption spectra, mutual orientation of the fluorophors and fluorescence quantum yield of the donor. In the case of a given R_0 the accuracy of the intensity measurements is another important parameter limiting the minimum amount of measurable transfer, which in turn limits the maximum detectable separation distance. In favourable cases it is possible to detect the energy transfer between donor-acceptor separated by a distance exceeding R_0 several times fold. Estep and Thompson (Biophys. J., 26:195, 1979) report a 5 % quenching of the donor (anthracene) fluorescence by the acceptor (perylene) at a separation of 28.9 nm (These authors define the separation as the square root of average area occupied per chromophore.)

One of the methods for the investigation of interactions between different cell surface elements is the blocking test. The inhibition of binding a ligand to the appropriate membrane component, A, caused by a preincubation with another ligand (binding to another membrane component, B) can be interpreted as an interaction between A and B. With this method interactions have been reported between I_a antigens and F_c receptors (Dickler and Sachs, J. Exp. Med., 140:779, 1974). H-2D^b and Lyt 2.2, H-2K^b and Lyt 1.2 (Boyse et al., Proc. Natl. Acad. Sci. USA, 60:886, 1968; Flaherty and Zimmerman, ibid, 76:1990, 1979). In a second method molecule A is allowed to cap by having bound an appropriate ligand and cocapping (the B molecule's moving together with A) is tested. With this method cocapping was found for surface IgM and lipopolysaccharide receptors

(Forni and Continho, Nature, 273:304, 1978). Unfortunately results of these investigations cannot be regarded as evidence for the existence of linked A and B species. Although this kind of interpretation is the simplest but not the only one.
The possibility to cap the H-2 antigens and Con-A receptors to the same pole of the cell cannot be regarded as an evidence for a real cocapping as both of them can cap independently leaving the disperse distribution of the other unaltered.

SZAMEL:

In contrast to your data a lot of recent evidence suggest that the H-2 antigen may contain Con-A binding sugar components. Please give comment!

TRÓN:

Nobody would deny the possibility of Con-A binding sugar components in the H-2 antigen. However our data show that the occupied Con-A receptors and the antibody-H-2 antigen complexes can be capped independently even at saturating conditions. The relatively high measured transfer efficiency between these receptors indicates some kind of interaction. The independent capping, however, argues against a firm complex between the donor and acceptor labeled entities. Our results are consistent with buried sugar components of the H-2 antigen not accessible for the Con-A.

DALE:

Our energy transfer experiments (Dale et al., in: Fluorescent Probes, 1981, pp. 159, Eds: G.S. Beddard and M.A. West, Academic Press, New York) on fluorescein- and rhodamine-labeled Concanavalin-A saturating Balb/c-3T3, 3T12 and SV3T3 fibroblasts, at 4^{o}C where there was no patching, carried out on suspensions in a cuvette, gave an efficiency of about 15 %. On a two-dimensional distribution model, about four-fold lower concentrations than those estimated from the amount bound and the cell surface

area would have given this result assuming a random distribution, and even less if the distribution were non-random, due to micro-clustering of the Concanavalin-A receptors for instance. In fact, about three-fold more Concanavalin-A was bound than could be accomodated in a monolayer on the cell surface area available. Furthermore, on patching at 37°C no change in energy transfer efficiency was observed, although the nominal two-dimensional concentration increase should have led to an increase in the energy transfer efficiency to about 70 % or more. These observations may be rationalized by comparison with electron micrograph data in the literature showing ferritin-labeled Concanavalin-A distributed three-dimensionally through a layer, presumably of glycoprotein, extending some 10 nm from the plasma membrane. Estimates of this thickness from the observed energy transfer efficiency, amount bound and cell surface area correspond reasonably well with this value. Furthermore, patching is seen from the electron micrographs to arise from folding over of the layer without increase in the concentration of the label rather than from lateral aggregation of the label in two-dimensions, consistent with the lack of change of transfer efficiency on patching. While the interpretation of energy transfer measurements between donors and acceptors bound to particular membrane proteins may be validly interpretable in terms of a two-dimensional distribution model, caution in interpreting such data for Concanavalin-A bound probes, and presumably also for other lectins, is indicated.

TRÓN:

As to our knowledge, there are no electron micrograph data on T41 cells. Appearingly our system differs basically from those of used in your experiments. The number of Con-A receptors per T41 cells (determined by digestion of samples of T41 cells saturated with labeled Con-A) was 4.4×10^6. Such an amount of Con-A molecules can be easily accomodated in a monolayer on the surface of a cell of 15 μm diameter.

As I understand, you applied a lower (1:1) acceptor to donor ratio than our one. We applied quite a few acceptor to donor ratios. The highest energy transfer efficiency (30 %) was gained at 4:1 acceptor to donor ratio.

MARTONOSI:

What are the estimated densities of H-2 and Con-A receptors on the cell surface?
Is it likely that orientative effects could influence (reduce) the transfer efficiency and therefore the calculated distances?

TRÓN:

The estimated density of Con-A receptors on T41 cells was $6 \times 10^3/\mu m^2$ assuming a smooth 15 μm diameter spherical cell. That of the H-2 antigens is roughly one order of magnitude lower.
You are right. Orientation effects result in a multiplication factor in the expression describing the interrelationship between the energy transfer efficiency and the separation distance. In the case of our system, however, the random orientation (K=2/3) is an acceptable assumption because of the random labeling of both donor and acceptor conjugated ligands.

SCHINDLER:

Lymphocytes can undergo large changes in their surface contours, e.g., in blebs, microvilli, micro-spikes, following lectin binding. How would this affect energy transfer experiments, particularly the categorization of aggregation?

TRÓN:

These effects can develop on the lymphocyte surface when the cells are incubated with lectins at 37°C. In our case the cells were labeled with Con-A at 4°C.
We have not observed blebs and any deviations from the smooth surface when investigating our labeled cells

under the microscope. There are no electron microscopic data of this kind available on T41 cells characterizing submicroscopic microvilli etc. As our cells were of T type the submicroscopic deviations are supposed to be less compared to B cells.

Surface contour parameters can effect the conclusions drawn from transfer efficiency measurements by at least two ways. Substantial deviations from the smooth surface increase the area the fluorophors can be distributed on. This leads to an underestimation of the average donor-acceptor separation distance calculated from the total number of binding sites and assuming a smooth spherical surface. This effect would decrease the probability "to find" an aggregated distribution, if the criteria of it is a smaller separation distance calculated from the measured value of the E compared to that of estimated from the total number of binding sites, assuming a smooth spherical surface. On the other hand, in the case of a rough surface the distance between two particular sites may be much less than that measured along the surface. If the separation between "adjacent membrane layers" of the same or neighbouring microvilli are within 10 nm the same numerical value of the transfer efficiency may be measured at larger separation distances compared to the smooth surface case.

This is a case with effect opposite to the one discussed before. It has to be mentioned, however, that this kind of geometry seems to be unlikely in our case.

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DESIGN OF REVERSIBLE PHOTOCHEMICAL CROSS-LINKERS IN CONJUNCTION WITH SPECIFIC IgG

APPLICATION IN STUDIES OF TOPOGRAPHICAL DISTRIBUTIONS OF VIRAL AND CELLULAR MEMBRANE PROTEINS AND IgG RECOGNITION OF LEFT-HANDED Z-DNA CONFORMATIONS

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I. INTRODUCTION

Reversible photo-chemical crosslinking reagents have been developed which can be used in conjunction with specific poly- or monoclonal IgGs to analyze dynamic protein, drug, toxin, and/or DNA structures. These techniques were initially designed to measure nearest-neighbor interactions in membranes, chromatin or other cellular organelles. They are also being applied to deliver proteins, radionuclides, drugs, genes, or toxins to specific sites. Reviews outlining progress in structural analyses using either cross-linking or immunological probes alone or in conjunction with other methods have been given by Staros, (1980); Jovin, (1979, 1980); Ji, (1979); Freedman, (1978); Schimmel and Budzik, (1979 and see other papers from that volume); Peters and Richards, (1979); Atassi, (1977); Song and Tapley, (1979); Chowdhry and Westheimer, (1979); Issacs *et al*, (1982); Gitler, (1980); Colman, (1983). Recent reviews on membrane structure, genetics and expression of histocompatibility antigens can be found in Mescher (1982) and other papers in that volume.

Table 1 summarizes the current techniques developed in our own and other labs for assessing topographical relationships between molecules using specific immunoglobulin probes. The problems often associated with cross-linking methods in conjunction with antibodies are also listed. However, the exquisite specificity of immunoglobulins, their wide range of binding affinities and reactivities under a variety of conditions, their ability to stabilize dynamic structures, and to be introduced onto or into living cells or organelles under physiological conditions offer important advantages for studying molecular structures, functions and dynamics (Table 1, C). Also, in conjunction with photo-activatable cross-linking agents, they can be easily used to establish topographical distributions at the level of individual molecules or chains.

Theoretically, it is possible to covalently link polypeptides, drugs, or polynucleotides that are adjacent to each other with bifunctional cross-linking reagents. The flexibility and the motion(s) of the structure can also be frozen. Complex structures comprised of several polypeptides and/or polynucleotide sequences can have functional groups which lie within different domains. These would have different distances between their constituent groups. Specific polypeptides or monospecific IgG probes with pre-attached heterobifunctional cross-linkers (possessing free photo-

activatable groups, or with attached polynucleotides, drugs, toxins, or polypeptides) can also be applied as site-specific reporters, substrates or probes to various systems (Table 1, D).

TABLE 1

Ig PROBES FOR MEASURING TOPOGRAPHICAL DISTRIBUTIONS

A. Techniques using IgGs for determining proximities between cell organelles (membrane, chromatin) and bound polypeptides, polynucleotides, drugs and other antigens

1. Immuno-electron, -reflectance, and -fluorescence microscopy
2. Antibody blocking tests
3. Co-capping and -patching assays
4. Co-purification, -electrophoresis, sequential immunoprecipitation or radioimmunoassay
5. Fluorescence resonance energy transfer
6. Time-dependent phosphorescence anisotropy
7. Chemical (defined length) and laser (without added chemical) light activated cross-linking

B. Problems associated with use of Igs to assess spatial relations

1. Antibody probes can cause artifacts due to aggregation and re-arrangement of antigens (eg. membranes)
2. Igs with higher binding affinities for free compared to complexed antigens can affect the dynamic equilibrium of certain complexes
3. Insufficient spatial resolution

C. Advantages of Ig probes

1. Availability of monospecific or monoclonal ligands
2. Cooperative and non-cooperative binding modes
3. High affinities; reversibility
4. Stabilization of transient or dynamic structures
5. Micro-injectable
6. Bi- (IgG) or polyvalent (eg. IgM), hybrid molecules constructable
7. Can be spectroscopically, biochemically or radioactively labelled

D. Biological Substrates Being Studied

1. Plasma, mitochondrial, viral and nuclear membranes
2. Virus, ribosomes and chromatin
3. Receptors (Ig, viral, T-cell, acetylcholine, nucleotide, hormone)
4. Enzymes, transport and packaging proteins, toxins, drugs

Cross-linked structures can be solubilized by treatment with detergents or salts and then analyzed by protein or DNA separation methods such as electrophoresis in gels of a restrictive medium. Usually the cross-linked complexes are separated in a first dimension with the links intact and then in a second dimension after reversal of the linkages. The structure of the complex can be deduced by identification of the cross-linked polypeptides or

polymers and their proximities defined by the span of the cross-linking reagent.

In practice, many traditional cross-linkers are labile, change or denature the structure being studied, are not compatible with physiological reaction conditions or require fairly long reaction times, and cannot be quantitatively dissociated. Thus, they give complex or artifactual results, especially with large or dynamic structures. Clearly, one of the most important recent advances has been the design, synthesis, and application of non-toxic, fast, efficient, stable and fully- reversible cross-linkers that do not denature their substrates, do not affect their recognition by other ligands, and do not alter the electrophoretic mobility of the polymer or polypeptide carrier such as to prevent its separation or confuse the determination of molecular weights.

A new heterobifunctional photo-activatable cross-linker was developed for use in conjunction with specific immunoglobulins in measuring protein neighbors to viral or cell surface polypeptides (Zarling *et al,* 1982). Subsequently, it became clear that the photo-activatable nitrene intermediate of this reagent could insert into nucleic acids and thus would be of particular interest in studies using specific IgGs recognizing dynamic nucleic acid conformations in synthetic polynucleotides and chromatin (Jovin *et al,* (1983b); Robert-Nicoud *et al,* (1982); Arndt-Jovin *et al,* (1983); Zarling *et al,* (1983). The design and use of these probes and some systems where they are being applied is summarized here.

II. SADP REACTION SCHEME AND CROSS-LINKING OF PROTEINS

We wanted a relatively short heterobifunctional reagent with an imidoester (amino reactive) group on one end and a photo-activatable aryl azide group on the other and with a disulfide-reversible group in between. Previous experience with sulfones reversible by high pH (Zarling *et al.,* 1980) suggested their applicability in the designs described here, but they would not be practical for studies with RNA or double-stranded DNA substrates. Table 2 summarizes some of the advantages and disadvantages of SADP as a cross-linking reagent.

TABLE 2

ADVANTAGES AND LIMITATIONS OF SADP AS A CROSS-LINKING REAGENT

A. ADVANTAGES

1. Easily synthesized, soluble and stable
2. Heterobifunctional and readily cleavable by reduction
3. SADP coupled virus or proteins are biologically active
4. Aryl azide group can be selectively photo-activated to the reactive nitrene and is not reactive under physiological conditions in the absence of light. [Cross-linking occurs only when the aromatic chromophore absorbs energy]
5. Nitrenes do not require a specific reactive group (can react with any amino acid residue) and can have short half-lives, estimated in the 0.1-1 ms range (triplet nitrenes in ethanol; Reiser *et al,* 1968) permitting distinction between stable versus collision complexes

6. Imidoester reacts stably under mild conditions with a high degree of specificity for amino groups in immunologlobulins. Interaction can be without any change on the net charge or reactivity of the antibody

B. LIMITATIONS

1. Reductant cannot be used to isolate complexes and disulfide exchange reactions must be monitored or prevented
2. Preference of aryl azide for nucleophiles
3. Actual lifetimes after photolysis may be longer under some conditions (Mas *et al*, 1980)
4. DNA chain breakage (Woolley and Dohrmann, 1983)

Bastos (1975) first described azide group-mediated reactions with DNA that are light activated. Replacement of ethidium amino groups with azide (Graves *et al*, 1977) permits photo-reactive cross-linking of double-stranded nucleic acids (Woolley and Dohrmann, 1983). Woolley and Dohrmann (1983) pointed out that it is not known where nitrenes insert into DNA because of lack of agreement as to exactly where polyaromatics are situated in DNA.

The scheme developed by Drs. P.K. Smith and E.K. Fujimoto (Zarling *et al*, 1982) for synthesizing the 11 Å SADP molecule is shown in Figure 1. The product is stable to long term storage and has been made commercially available (Pierce Chemical Company).

SADP REACTION SCHEME

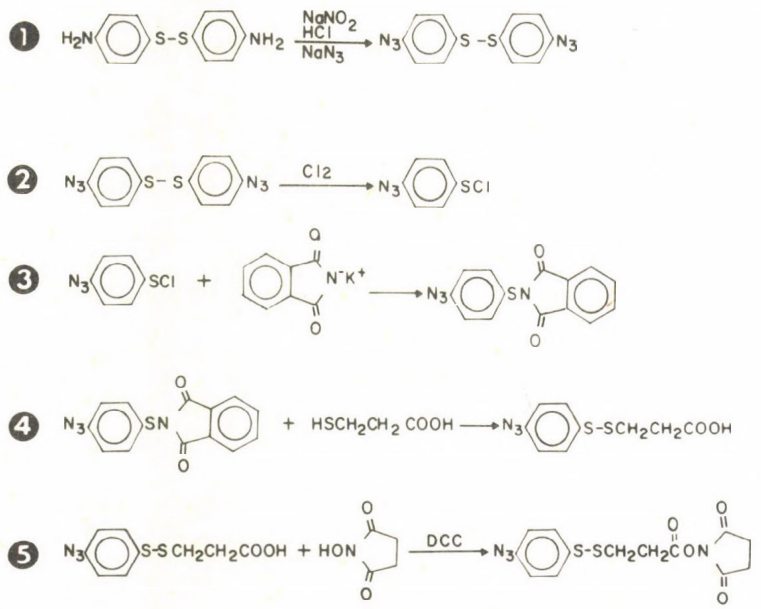

Fig. 1. A) SADP reaction scheme. SAPD, N-succinimidyl 4-azidophenyl-1, 3'-dithiopropionate was prepared according to a reaction scheme (shown) which generally follows modification of methods developed by Kiehm and Ji (1977) and others (Mikkeslsen and Wallach, 1976; Smith and Brown, 1951; Behforouz and Kerwood, 1969; Kilvenyi *et al*, 1955; Harpp *et al*, 1970; Anderson *et al*, 1964). Starting from 4-aminophenyl disulfide (Aldrich Chemical Co.), the five step synthesis requires the following intermediates: 4,4'-dithiobis-phenylazide (Pierce Chemical Co.) (Step 1) (Mikkeslsen and Wallach, 1976; Smith and Brown, 1951), 4-azidophenylsulfenyl chloride (Step 2) (Behforouz and Kerwood, 1969), n-(4-azidophenylthio)phthalimide (Step 3) (Behforouz and Kerwood, 1969; Kilveneyi *et al.*, 1955), and (4-azidophenyl)-1,3'-dithiopropionic acid and N-hydroxysuccinimide in the presence of N,N'-dithiopropionic acid (Step 4) (Harpp *et al*,. 1970). SADP is then prepared from (4-azidophenyl)-1,3'dithiopropionic acid and N-hydroxysuccinimide in the presence of N,N'-dicyclohexylcarbodiimide in methylene chloride (Step 5) (Anderson *et al.*, 1964). The isolation of crude product and recrystallization from methyl chloride-hexane (1:4 v/v) gives a very pale yellow crystalline material (66% yeild), m.p. 90-91° C. The infrared and nuclear magnetic resonance spectra are consistent with the expected structure of SADP.

The cross-linking of two proteins P_1 and P_2 with SADP is shown schematically in Figure 2.

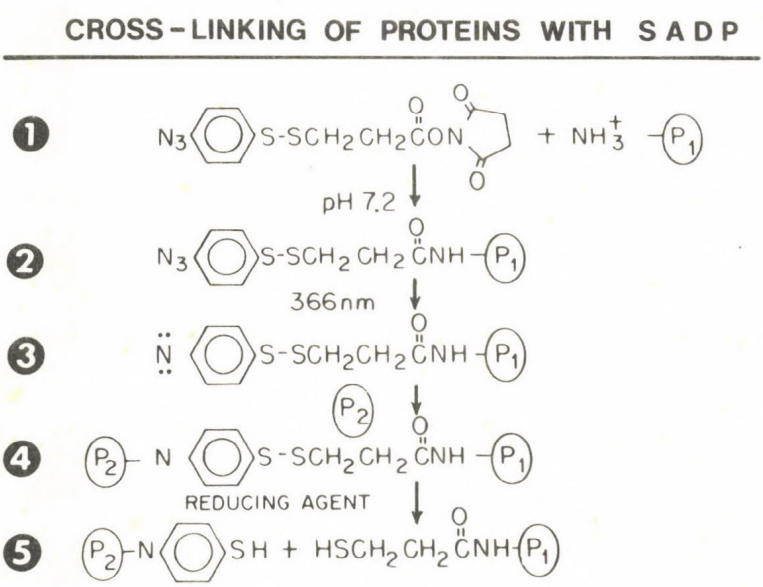

Fig. 2. Reaction of SADP to a protein, P_1, via an amino group and subsequently to a protein, P_2, via an azide group. Cleavage of the internal disulfide in the SADP molecule occurs after reduction.

To test SADP, experiments were performed to introduce specific proteins which were pre-coupled to the amino-reactive end (imidoester) of SADP into the membranes of living cells. The basic design of this experiment is outlined in Table 3.

TABLE 3

SCHEME OF SADP EXPERIMENTS DESIGNED TO MEASURE NEIGHBORING STRUCTURES TO:

A. SADP-Derivatized Proteins Inserted Into Membranes (eg. viral)

1. Sendai virus is mixed with SADP in the dark so the amino-reactive end will bind to the virion envelope glycoprotein spikes, HN (Haemagglutinin, Neuraminidase) and F (fusion protein). The photo-activatable end (*) remains free and light-protected.
2. Cloned mouse P815 mastocytoma cells ($H\text{-}2^b$, DBA/2 mouse) are surface iodinated with ^{125}I.
3. In the dark, ^{125}I-labelled P815 cells are infected with SV-SADP-*. Viral glycoproteins insert into the cell membrane.
4. Infected cells are exposed to 366 nm UV light to activate the free (*) end of SADP and envelope proteins are cross-linked to their neighbors.
5. Cell membrane extracts (non-ionic detergent lysis) are immunoprecipitated with polyclonal or monoclonal anti-Sendai virus envelope glycoprotein serum (anti-HN, anti-F serum) so that ^{125}I-labelled cell proteins (X) which may be cross-linked to SV protein (^{125}I-X-SADP-Sendai-Ig) will be selected.
6. Ig selected with *Staphylococcus* bearing Protein A and ^{125}I-labelled proteins analyzed by gel electrophoresis.

B. SADP-derivatized IgGs Attached To Their Substrates (eg. Z DNA)

1. Polyclonal or monoclonal anti-Z DNA IgG is mixed with SADP in the dark (as above) to form anti-Z IgG-SADP*.
2. Synthetic Z DNA polymers or supercoiled DNA (eg. SV-40 intracellular DNA) are radiolabelled [^3H-thymidine] or chromatin (eg. SV-40 minichromosomes) proteins are radioiodinated (^{125}I, tyrosine).
3. In the dark, labelled DNA or chromatin substrates are bound by anti-Z IgG-SADP* or cell nuclei are micro-injected.
4. Ig-DNA complexes are exposed to UV light (as in A above) to form covalent linkages.
5. Ig-SADP-protein or Ig-SADP-DNA are selected with Protein A or anti-globulin.
6. Protein and/or DNA cross-links analyzed by gel electrophoresis.

III. SADP COUPLING TO VIRAL OR CELLULAR PROTEINS

A. Retention of Biological Activity

Sendai virus was coupled to SADP at various concentrations and the effects on viral activity were measured. In these experiments the SADP molecule was photoactivated and allowed to decay to prevent any possible reactivity of the azide group (Table 3). This one-armed reagent was coupled

to virus and the derivatized virus was fused into the membranes of mouse cells, which were then tested as targets for recognition by anti-Sendai-specific cytolytic-T-cells (Zarling *et al*, 1982).

At a molar ratio of 1 SADP molecule per 1 Sendai protein (calculated using the average M_r of 50,000 daltons per molecule) approximately 80% of the activity of the virus remained (Table 4).

TABLE 4

EFFECTS OF SADP COUPLING TO SENDAI VIRUS[1]

[SADP] μM	Molar ratio of SADP/viral protein	relative activity[2]
0	0	1.0
2	0.1	.9
20	1	.8
100	5	.3
500	25	.02

1. Purified Sendai virus was coupled with SADP that had been photoactivated and allowed to decay (see text). Coupled virus was fused into P815 cells (Table 3) at a concentration of .01 μg virus/1000 cells. Cells were subsequently labelled and tested for their ability to be lysed (lytic units) by syngeneic (Balb/c) anti-Sendai virus-specific cytotoxic-T-cells (CTL). Additional details are given in Zarling *et al*, 1982.
2. Data normalized relative to 60 lytic units per 10^6 cells sensitized by SADP coupled virus.

Increasing the molar ratio to 5 SADP molecules to 1 protein molecule yielded virus which retained 30% of the control activity. This SADP concentration was selected for experiments in which Sendai virus proteins were tested for their ability to cross-link to one another via the active bifunctional reagent.

B. Cross-linking of virus envelope glycoproteins in homo-molecular complexes.

Sendai virions were surface-radioiodinated with ^{125}I (Iodo-gen, Pierce; 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril), coupled with SADP in the dark, and exposed to UV light. A 120,000 dalton polypeptide was observed, which after reduction gave 47,000 and 15,000 dalton polypeptides (Table 5). The 47,000 and 15,000 dalton proteins are the two subunits (F_1 and F_2, respectively), of the virion, fusion glycoprotein ($F_0 = F_1 + F_2$, for discussion see Zarling *et al*, (1982) and Markwell and Fox, (1980)). Thus, SADP could detect an apparent tetramer, comprised of two F_1 and two F_2 polypeptides within 11 Å of one another.

The F glycoprotein is the spike observed on the surface of the virion which possesses an activity promoting fusion of viral and cellular membranes

Homma and Ohuchi, (1973); Scheid and Choppin, (1974). In other experiments, we have shown that the Sendai fusion activity is essential for formation of the target antigen recognized by cytotoxic T-cells (Table 5) on Sendai-infected mouse cells (Sugamura *et al*, 1977).

Similarly, in experiments using a 13 Å base-cleavable homobifunctional crosslinker, bis-[2 (succinimidooxycarbonyloxy) ethyl] sulfone, BSOCOES. we found the envelope glycoprotein of murine leukemia virus budding from constitutively infected and transformed mouse leukemia cells (Table 5) in homo-trimeric or -dimeric complexes.

TABLE 5

COMPOSITIONS OF SOME SADP OR BSOCOES CROSS-LINKED POLYPEPTIDES

Linker & Substrate	Ig Probe	APPROXIMATE M_r WITH: Links-intact	Links-removed	Proposed Structure	Proposed Function
SADP-Sendai virion	none	120,000	47,000+15,000	Dimer, F_0	Spike, fusion
" infected P815 ($H\text{-}2^b$)	anti-SV[1]	HMW[2]	45,000+17,000	Unknown, not $H\text{-}2^b$	Host receptors for virus or cytoskeletal element ?
BSOCOES-RBL-5 ($H\text{-}2^b$)	anti-MLV gp70[3]	210,000	70,000	Trimer, gp70	Envelope knobs, fusion
		140,000	70,000	Dimer, gp70	
"	anti-$H\text{-}2^b$[3]	58,000	46,000+12,000	Dimer, $H\text{-}2^b$ heavy and light chain	recognizes self & viral or other neoantigens
"	"	35,000	46,000	$H\text{-}2^b$ heavy chain (intra-chain linked)	"

1. Sendai virus envelope glycoproteins were coupled to SADP as described in Table 3 A and fused into P815 cells. Immunoprecipitates were formed with equine polyclonal anti-SV.
2. High molecular weight polypeptides not resolved by SDS-PAGE.
3. BSOCOES was used to cross-link radioiodinated surface polypeptides on tranformed and constitutively-producing Rauscher MLV C57BL/6 mouse cells as described in Zarling *et al*, (1980). Immunoprecipitates were formed with polyclonal caprine anti-MLV gp70 Ig.
4. Immunoprecipitates formed with murine alloantisera.

MLV gp70 also possesses a fusion activity (Zarling and Keshet, 1979). The envelope gene of a human T-cell leukemia virus is reported to have significant homology with HLA antigen nucleotide sequences (Clarke *et al*, 1983). The cell lines which produce this virus also have altered HLA expression (Mann *et al*, 1983). The fusion activity associated with the envelope glycoprotein of MLV, like Sendai virus, is essential for the infectivity of the virion, for formation of the CTL target antigens in virus-infected cells or on some transformed cells recognized by syngeneic virus-immune CTLs (Watson, *et al*, 1979; Zarling and Keshet, 1979).

C. Nearest neighbors to Sendai envelope glycoprotein antigens after fusion into surface membranes of newly infected cells: lack of association with class I mouse histocompatibility antigens; association with 17,000 or 45,000 dalton host cell surface polypeptides.

No evidence exists from SADP cross-linking experiments (Table 5) that Sendai envelope glycoproteins become associated with H-2 antigens at the time of CTL target antigen formation. Similarly, using BSOCOES cross-linking the major envelope glycoprotein of MLV exists in homo-dimeric and trimeric associations, but no clear evidence could be found for bimolecular gp70/H-2 cross-linked products. A discussion of the apparent lack of association of H-2 antigens with some Sendai or other viral antigens as evidence against a single receptor model for the general structure of CTL receptors is given in the participants' remarks section (see following section and also Calafat *et al*, 1981). However, many details of the *in vivo* structure of dynamic H-2 antigens are lacking.

Interactions between mouse or human major histocompatibility (MHC) complex (mouse, H-2; human, HLA, etc.) antigens and certain viral (eg. human adenovirus type 2 E19K, a viral-coded 19,000 dalton glycoprotein synthesized in adenovirus-transformed rat cells or infected human cells, Kämpe *et al*, 1983; Pääbo *et al*, 1983) or human MHC antigens and epidermal growth factor (Schreiber *et al*, 1983) would suggest that some receptors may detect closely associated (MHC and neoantigen) molecules.

SADP may be generally useful in studies of the structure of these or cellular receptor-like or cytoskeletal polypeptides which can associate with newly inserted viral proteins (eg. the P815 cellular polypeptide of apparent M_r of 17,000 daltons which associated with Sendai envelope glycoproteins, Table 5). It would be interesting to use SADP in cross-linking studies between cells (eg. T-cell receptor on CTL in conjugation with target cells) or conjugates between cells and synthetic vesicles containing SADP-bound viral (etc.) antigens. For discussion see also Berke, (1983).

The cross-linking experiments which suggest that the most frequent topographical distribution of $H\text{-}2^b$ antigens are within simple dimeric associations between one heavy (46K) and one light (12K), β2-microglobulin, chain (Table 4) indicates that there is no significant homo-clustering of H-2 heavy chains on the cell surface.

Using fluorescence-resonance energy transfer with a dual laser flow sorter, Damjanovich *et al* (1983) have shown murine monoclonal anti-H-2 K^k IgG probes do not detect appreciable clustering of heavy chains on the cell surface. Time-resolved phosphorescence anisotropy measurements using these

monoclonal probes also show rotational mobilities reflecting predominant uniaxial modes of monomeric antigens (Damjanovich *et al*, 1983).

D. Immunogenicity of SADP cross-linked polypeptides

The finding of mouse cell polypeptides (eg. the 17,000 dalton protein described in Table 5), which could be cross-linked to Sendai envelope proteins suggested that these cellular proteins might affect the immunogenicity of the viral antigens. SADP-Sendai-cellular proteins were prepared (as in Table 3, Table 5) selected by immunoprecipitation with anti-Sendai Ig and *Staphylococcus* bearing Protein A (Kessler *et al*, 1975) and antigen-antibody complexes were dissociated using 3.5 M $MgCl_2$. The $MgCl_2$ was dialyzed and the immunogenicity of cross-linked polypeptides was compared with non-cross-linked controls. No differences in their immunogenicities were detected (Zarling *et al*, 1982) and thus a highly antigenic complex of viral and host proteins appears unlikely.

IV. ANTIBODIES TO DNA AS PROBES OF DNA DYNAMIC STRUCTURE AND FUNCTION. Ig6 CROSS-LINKING OF DNA

A. Immunogenicity of base or phosphate backbone modified left-handed Z DNA permitting an immunological analysis of left-handed DNA conformations.

B DNA is not immunogenic, whereas Z DNA, for reasons that are not understood, is highly so. For review see Lafer *et al*, 1983 and Zarling *et al*, 1983. We have compared the members of the poly[d(G-C)] family which are either "constitutively" or "facultatively" (Jovin *et al*, 1983b) in left-handed conformations (Table 6). All of the left-handed Z DNAs tested were highly immunogenic in rabbits and mice.

TABLE 6

IMMUNOGENICITIES OF LEFT-HANDED Z AND RIGHT-HANDED B DNAS[1]

POLYMER	IMMUNOGENICITY	REFERENCE
Z DNA		
Constitutive[2]		
br-poly[d(G-C)]	+	Lafer *et al*, 1981
poly[d(G-br^5 C)]	+	Zarling *et al*, 1983a,b
poly[d(G-io^5 C)]	+	McIntosh *et al*, 1983b; Zarling *et al*, 1983a,b
Facultative[3]		
poly[d(G^{Br}C)]	+	Jovin *et al*, 1983a,d; Zarling *et al*, 1983a
AAF-poly[d(G-C)]	+	Hanau *et al*, 1983
dien-Pt-poly[d(G-C)]	+	Malfoy & Leng, 1981; Malfoy *et al*,1982
poly[d(G-m^5 C)]	+	Lafer *et al*, 1983; Zarling *et al*, 1983a

B DNA		
Constitutive[4]		
poly[d(C^{Br}-G)]	-	Zarling *et al*, 1983a

1. Rabbits and mice were immunized with synthetic polynucleotides as described in references. Ig was tested by radioimmunoassay (RIA) on the left-handed Z form of poly[d(G-C)] in 4.0 M NaCl. +, reactive; -, non-reactive. References are given only to immunogenicity, see also Jovin *et al*, 1983d for other references to Z structures of these polymers.
2. Exists in the Z state under all tested ionic conditions
3. Exists in the Z state under some physiological conditions
4. No B to Z transition occurs under tested conditions and is constitutively in the B state (Jovin *et al*, 1983a,d).

Thus, both constitutive and physiological Z DNAs are immunogenic whereas constitutive B DNA (or other physiological B DNAs) such as poly[d(C^{Br}-G)] are relatively non-immunogenic (Zarling *et al*, 1983). We also find that some anti-Z IgGs induced by poly[d(G-C)] derivatives can recognize the left-handed structures of poly[d(A-C)·d(G-T)] derivatives (McIntosh *et al*, 1983). Certain other anti-Z IgGs do not recognize the poly[d(A-C)·d(G-T)] sequence, its modified derivatives, or certain modifications in poly[d(G-C)] (McIntosh *et al*, 1983; Zarling *et al*, 1983a,b; Jovin *et al*, 1983d).

B. Antibody recognition and stabilization of transient Z DNA structures.

The anti-Z IgGs not only recognize salt- or thermally-stabilized Z conformations and sequences but can stabilize dynamic Z structures. For example, when Z polynucleotides are bound by anti-Z IgGs in high salt and then the IgG-DNA complexes are diluted into low salt to induce a reverse Z→B transition, all the molecules still have a Z region detectable by RIA.

Furthermore, transient Z structures in a B-Z equilibrium can be stabilized with these various anti-Z IgGs, as summarized in Table 7.

TABLE 7

ANTI-Br-POLY[d(G-C)] IgG STABILIZATION OF DYNAMIC Z STRUCTURES

1. Dilution of IgG-Z DNA complex formed in high salt (eg. poly[d(G-C)]) into low salt does not remove IgG.
2. In the Z state (4.0 M NaCl; transition midpoint =2.3 M NaCl) all DNA substrate molecules score as RIA-positive after ≤ 5 seconds of IgG binding at 53° C ($k_b > 10^5$ $M^{-1}s^{-1}$).
3. In 1.5 M NaCl at 53° C the kinetics are slower:
   a. reaction half-time of 40 minutes
   b. all DNA bound by 80-100 minutes
4. Reaction rates increase with increasing IgG concentration; anti-Z IgG perturbs the B-Z equilibrium in favor of the Z conformation.
5. Experiments with poly[d(G-m^{5}C)] in 0.4 M NaCl (transition midpoint

=1.2 M NaCl) show similar kinetics and dependence on IgG concentrations, on temperature, and on another anti-Z IgG probe with a specificity requiring a cytosine C-5 bulky group substitution (eg. anti-poly[d(G-br^{5}C)], and see Zarling *et al*, 1983a,b; Jovin *et al*, 1983d for experimental details).

In summary, the IgGs perturb the B-Z equilibrium as a function of IgG specificity, concentration, time, DNA sequence, and length, ionic state, temperature and topological state (see next section). However, the IgGs by themselves are not able to recognize B DNA or catalyse the B→Z transition.

During the past several years we have sought to determine whether Z conformations exist in natural sequences and if so, what natural factors (eg. environmental or host cellular polypeptides, enzymes, etc.) stabilize their occurrence or catalyze their formations. Anti-Z Ig probes can be used as models (eg. competitors) for Z DNA-binding proteins (for isolation and characterization see Nordheim *et al*, 1982 or, for discussion Zarling *et al*, 1983a) as well as probes for synthetic and natural DNA structure(s).

C. Anti-Z Ig cross-linking of natural Z DNA sequences in negatively supercoiled plasmids and viruses

Covalently closed circular plasmid and viral DNAs at their normal extracted negative superhelical density were tested as substrates for anti-Z IgGs in an electrophoretic assay. A typical experiment is shown in Fig. 3.

TABLE 8

ANTI-Z IgG RECOGNITION AND CROSS-LINKING OF NEGATIVELY SUPERCOILED DNAS

---

1. Assay by retardation of electrophoretic mobility of IgG-DNA complexes or blocking of restriction endonuclease cleavage sites.
2. Negative supercoiled Form I DNAs (pBR322, pBR328, pColEI, ϕX-174, M-13, SV-40, etc.) at their extracted densities react but relaxed Form II (treated with calf thymus topoisomerase I or bacterial gyrase minus ATP) or linear (present in DNA preparation as Form III or linearized by restriction endonuclease) forms do not.
3. Antibody-binding to DNAs is dependent on salt, temperature, time, DNA superhelical density, and IgG specificity and concentration.
4. Higher cross-linked (eg. trimers etc.) oligomers of some DNAs complexed with certain IgGs (eg. anti-br-poly[d(G-C)] and SV-40 or pBR322) are formed, and thus at least two left-handed Z DNA sites are present. Other IgGs with different specificities can detect single sites (eg. anti-poly[d(G-br^{5}C)]).
5. Restriction endonuclease sites are specifically blocked by IgG with or without chemical cross-linking.
6. Sequence-specific anti-Z IgGs differentially bind to different sequences in natural DNAs.
7. Sequence-specifity of anti-Z IgG on synthetic polynucleotides is maintained in IgG cross-linked complexes with corresponding inserts cloned into plasmid DNAs (Kilpatrick *et al*, 1983).

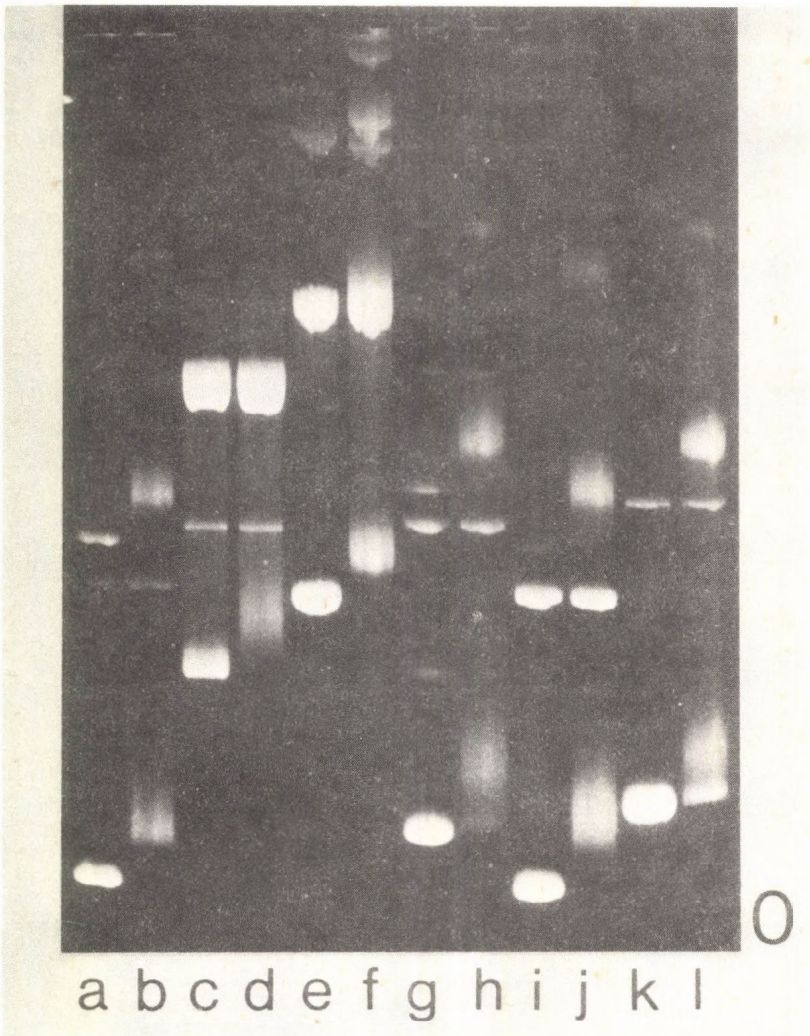

Fig. 3. Binding and cross-linking by anti-br-poly[d(G-C)] IgG of negatively supercoiled DNAs. DNAs (0.4 μg) were reacted at room temperature with 6.1 μg (lanes a, c, e, g, i, k) or 0 μg (lanes b, d, f, h, j, l) of purified rabbit T-4 anti-br-poly[d(G-C)] IgG (approximately 2% specific) and analyzed by electrophoresis in agarose gels. The DNAs used were: SV-40 (lanes a and b), pBR322 (prep. B, lanes c and d; prep. A, lanes k and l, note that this prep. contains small amounts of a double length DNA dimer), pBR328 (lanes e and f), pKSV10 (lanes g and h), pColE1 (lanes i and j). Note that in each case only the supercoiled Form I DNAs and not Form II DNAs specifically bind or are cross-linked by the anti-Z IgG. O, origin.

Furthermore, the binding of anti-Z IgG to supercoiled DNA can block specific restriction endonuclease cleavage sites with or in somes cases without cross-linking (unpublished experiments, Table 8 and see also Zarling *et al*, 1983a,b; Nordheim and Rich, 1983). However, nicked (Form II), linear (Form II) or topoisomerase-relaxed DNA (eg. SV-40 or pBR322) do not show any electrophoretic retardation (Figure 3, summarized in Table 8).

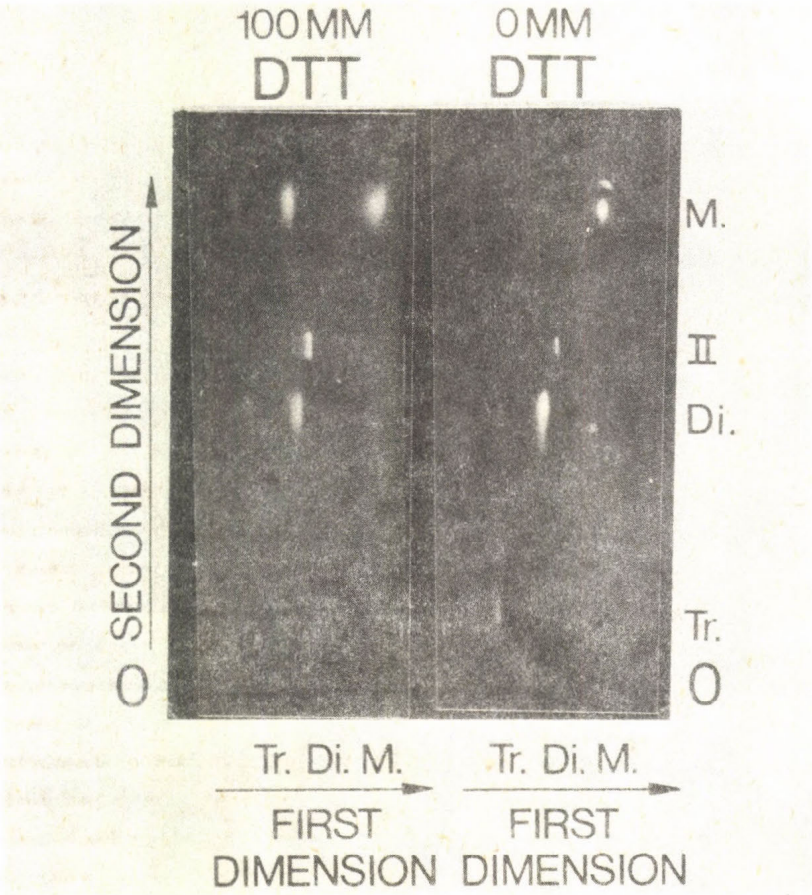

Fig. 4. DTT reduction (left) of IgG cross-linked viral DNA complexes. T-4-IgG-SV-40 DNA cross-linked complexes were separated in a first dimension (bottom) as Tr. (Trimer), Di. (Dimer) and M. (Monomer). The first dimension gel was treated with 100 mM (left) or 0 mM DTT (right) and then analyzed by electrophoresis in a second dimenson. Note that DTT (1hr, 24° C) reduces the IgG-DNA trimer and dimer to monomeric species. Data from Zarling *et al*, (1983a). O, origin in second dimension electrophoresis.

Thus, cross-linking of DNA molecules as multimers (dimers, trimers, and higher oligomers) occurs with bivalent IgGs. These assignments have been made from two-dimensional analysis of Ig cross-linked complexes (Figure 4). However, cross-linked species do not form with monovalent Fab fragments;

which can retard the electrophoretic mobility of Form I (but not Form II) DNAs. The Ig-DNA cross-linked complexes (eg. trimers and dimers) can be reduced to monomers by reduction of the immunoglobulin disulfide bonds with DTT (Nisonoff *et al*, (1975); Figure 4, Table 9 and Zarling *et al*, 1983a).

TABLE 9

ANTI-Br-POLY[d(G-C)] IgG BINDING OR CROSS-LINKING OF SV-40 DNA

	ELECTROPHORETIC MOBILITY IN:		
Ig Probe	First dimension with Ig added	Second dimension with Ig cross-links reduced with DTT	Proposed Structure
SV-40 FORM I NEGATIVELY SUPERCOILED SV-40 DNA (VIRION)[1]			
IgG[2]	retarded monomer	monomer	Ig-bound Form I
"	cross-linked dimer	monomer	bivalent IgG bound to two Form I DNAs
"	cross-linked trimer	monomer	bivalent IgG bound to three Form I DNAs
Fab frag.	retarded monomer	not done	Fab-bound Form I
SV-40 FORM II OR III NON-SUPERCOILED DNA (VIRION OR INTRACELLULAR)[1,2]			
IgG	non-retarded Form II	Form II	unbound Form II
"	non-retarded Form III	Form III	unbound Form III
Fab[3] frag.	non-retarded Form II	not done	unbound Form II
SV-40 FORM I IN A 75-90 S "MINICHROMOSOME" (INFECTED NUCLEI)[4]			
IgG	retarded minichrom. ? cross-linked	Form I	Ig bound to DNA in chromatin

1. Second dimension electrophoresis in 0.7% agarose gels was done after removal of IgG cross-links by reduction of interchain disulfide bonds with DTT.
2. Results from SV-40 virion DNA extracted from purified virus and or from the Hirt (1967) supernatant fraction. Binding to metabolically radiolabelled SV-40 Form I DNA is completely inhibited by addition of excess unlabelled synthetic Z DNA.
3. Fab binding experiment only performed with virion extracted SV-40 strain 776 DNA (BRL).
4. SV-40 minichromosomes (75-90 S) isolated from α-amanitin (Boehringer) treated nuclei bound to anti-Z IgG were analysed by electrophoresis in a first dimension 0.5% agarose gel and then were treated for 30 minutes with 2.5% SDS in 1x Tris-HCl-EDTA-Borate electrophoresis buffer prior to electrophoresis in a second dimension 0.5% agarose gel. DTT not used in analysis.

We have also tested [^{3}H]-thymidine-labelled SV-40 intracellular DNA intermediates and minichromosomes as substrates for anti-Z IgGs in direct RIA and in direct or competition electrophoretic assays. The intracellular SV-40 Form I but not Form II or III DNA is bound by anti-Z IgG (Table 9). The anti-Z Ig binding to SV-40 Form I DNA was competed completely with synthetic intracellular (constitutive) Z-poly[d(G-br^{5}C)] in a quantitative electrophoretic assay (Zarling *et al*, 1983b). Similarly, 75-90 S SV-40 minichromosomes (containing Form I SV-40 DNA) purified from the nuclei of infected monkey cells are bound by anti-Z IgG (Table 9).

Thus, it appears that Z DNA conformations can exist in native chromatin. See also Arndt-Jovin *et al*, (1983); Zarling *et al*, (1983a,b); Robert-Nicoud *et al*, (1982); Jovin *et al*, (1983b,c,d) for Z DNA in acid deproteinized or non-fixed polytene chromosomes. It is not known whether these left-handed regions are expressed transiently or are already stabilized by supercoiling, proteins and other factors. SADP-cross-linking experiments to detect anti-Z Ig neighbors could reveal topographical distributions of Z DNA-binding proteins and/or various histones (see also Russell *et al*, 1983 for discussion) phased on dynamic structures. Perhaps some of these may be compartmentalized on membranes, either directly or via membrane-bound polypeptides (for discussion see Schindler, 1983 and Schindler *et al*, this Symposium). It will also be interesting to study the biophysical and biological consequences of contact between various DNA conformations and membranes with resultant changes in cooperativity (Brosius *et al*, 1983).

V. SOME FUTURE DIRECTIONS OF CROSS-LINKING TECHNIQUES AND IMMUNO-PHOTO-CHEMICAL REACTIONS

A. Photo-chemical cross-linking reagents in site-directed cross-linking schemes.

Techniques have recently been developed for placing dHg^{5}CTP into unique restriction sites and then attaching psoralen to these sites via a Hg-S linkage (Saffran *et al*, (1983)) using the heterobifunctional cross-linker SPDP, N-succinimidyl-3-(2-pyridyldithio) propionate (Carlsson *et al*, 1978). Similar schemes using aryl azide cross-linkers with a reducible disulfide and Hg-S linking could (via disulfide exchange at pH 7.8) produce site-directed aryl azide groups. These site-directed probes may be useful for independently measuring sequence-specific DNA-binding and other proteins controlling gene expression.

The use of oligonucleotide probes containing a photoreactive aryl azide connected to the 5'-phosphate by a S-P bond is currently being developed by Meares and co-workers to identify RNA transcription complex pathways on linear DNA templates (Hanna and Meares, 1983a,b; Dr. C. Meares, personal communication). The S-P linkage is a particularly useful linkage because cross-links can be selectively reversed by phenylmercuric acetate at room temperature and neutral pH.

We have been particularly interested in transcription of different DNA (eg. B versus Z) conformations (Durand *et al*, 1983). Nucleotide probes with phororeactive aryl azides can be used to study linkages to both the T-7 phage DNA template and *E. coli* RNA polymerase β and α subunits (Hanna and Meares, 1983a).

B. Laser cross-linking of protein-DNA complexes without added chemical spacers and trapping of very short-lived conformational intermediates

Recently Harrison *et al* (1982) have used a single 120 mJ, 20 ns pulse at 248 nm from an excimer laser to cross-link *E. coli* RNA polymerase to phage T-7 DNA. The photochemistry of the laser cross-linking is just being revealed. It will be interesting to use selective modification (eg. heavy atoms) of the DNA bases or of the phosphate backbone (eg. phosphorothioate) in conjunction with laser cross-linking to look at the photochemical reactions at specific sites. A single-photon excitation is hypothesized. Analysis of the reaction curves with reductant (mercaptoethanol) as a scavenger of free radicals suggests at least two reactive species. One is believed to contain free radicals quenched by mercaptoethanol and another is not quenched (eg. long life-times or protected).

The laser cross-linking opens up the possibility of measuring several proteins in a large complex without introducing chemical spacers. Furthermore, it may allow cross-linking of short-lived DNA intermediates in the B-Z transition which are influenced by the enviornment (eg. ions, temperature, see Jovin *et al*, 1983d for review) or catalyzed by enzymes (eg. DNA topoisomerase I, etc. with supercoiled DNA, Zarling *et al*, 1983b).

Other experiments have shown that Z DNA serves as a template for prokaryotic or eukaryotic RNA polymerase II and that the anti-Z IgGs described here specifically inhibit transcription of Z but not B DNA (Durand *et al*, 1983). It will be possible to study the cross-linking of *E. coli* or wheat germ RNA II polymerase to synthetic or natural Z templates using laser induced cross-links, oligonucleotides with aryl azide groups, and photoactivatable chemical cross-linkers like SADP in conjunction with specific Ig probes.

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DISCUSSION

BHARGAVA:

In all the DNAs tested so far using the specific immunoglobulin technique, how much of the DNA sequence has turned out to be in the Z form?

Is it possible that your antibodies distinguish between the side-by-side model of DNA and the classical structure?

I am interested in the potential Z-DNA forming sequences in the LTR's.

ZARLING:

Dr. Donna Arndt-Jovin (Arndt-Jovin et al., 1983) in Göttingen has made photon counting measurements to estimate the amount of anti-Z IgG bound to left-handed regions of acid-fixed polytene chromosomes. The IgG occupancy calculated from saturation binding curves varies between 1 antibody/15 000 basepairs to 1 antibody/3000 basepairs in the bands with the highest density in the telomeres (Arndt-Jovin et al., 1983; Jovin et al., 1983b; Zarling et al., 1983a). In some negatively supercoiled plasmids or viral DNAs about 1 to 3 antibodies can be bound per 4000 to 5000 basepairs. So, the measured frequency of Z-DNA can range from about 0.01 % to 0.6 %. Also, some of these structures could exist in a transient Z state. The anti-Z antibodies specifically bind left-handed Z-DNA helices and not the corresponding classical B forms. It is most likely that the solution (e.g. high salt or supercoil stabilized) Z forms have structures similar to those in the crystal.

There aren't any experimental data yet that show sequences within a long terminal repeat (LTR) from a Retrovirus can adopt a stable Z configuration. The LTR region of certain Retroviruses contains alternating purine/pyrimidine sequences with the potential forming Z-DNA. In September at the John Innes Symposium on the Biological

Consequences of DNA Structure, Dr. Anton Berns (The Netherlands) and I organized experiments to determine if various anti-Z IgGs could stably bind to cloned LTRs whose potential Z sequences were induced by supercoiling.

SZABÓ:

How do your data relate to the dual recognition hypothesis?

ZARLING:

In the dual recognition model, two different CTL receptors would recognize the viral and H-2 polypeptides as separate molecules. The dual recognition hypothesis doesn't require that the two antigens be close each other, whereas the altered-self model does. The data showing that Sendai envelope glycoproteins are unlikely to be associated with H-2K/D polypeptides suggest that any altered-self model should be very special indeed. For example, physical association might occur only during the interaction of the CTL receptor either with the viral or cellular antigens and then lysis might take place after the contact formation between the two antigens. The demonstration of the unliklihood of the altered-self model for any virus-cell system would suggest that the model is generally unlikely. So, even in the case when viral and H-2 antigens are in close proximity it is possible that CTL lysis occurs using two receptors (dual recognition). I'd like to emphasize that the SADP-derivatized virus must be useful in the isolation of receptor(s) on CTLs which recognize viral antigens on infected cell surfaces. For example a covalent cross-linkage might be formed between a viral glycoprotein in the cellular membrane and the CTL receptor on the effector cell.

SCHINDLER:

Are there any antibodies against Z-DNA found in Lupus patients?

ZARLING:

Yes, anti-Z-DNA antibodies can be found in human sera from Lupus patients (Lafer et al., 1983; for discussion see Zarling et al., 1983). In collaboration with Dr. T. Trost at the clinics of the University of Cologne, we found anti-Z antibodies in the serum from a Lupus patient but not in normal human serum. In other experiments with Lupus mouse sera from Dr. H. Kolb's colony at the University of Dusseldorf I found anti-Z antibodies in the sera of many Lupus mice of certain ages. Dr. E. Lafer in Boston first showed anti-Z antibodies in MRL/1pr mice with Lupus disease (Lafer et al., 1981, 1983).

TIGYI, G.:

What is the size of an antigenic determinant in molecular terms of an anti-Z DNA monoclonal antibody recognition site?

How many anti-Z DNA antibody producing clones could you find from one fusion?

ZARLING:

The combining site of the anti-Z DNA IgG can span about four nucleotides. A 1.5x2 nm antibody binding site can be located on the convex surface (e.g., C-5 position of cytosine) or possibly on the phosphate backbone.

I fused the spleen cells from a serologically positive mouse with PAI tumor cells and obtained 22 anti-Z-DNA IgG producing hybridomas after screening approximately 800 clones; thus about 3 % of the clones could be scored as anti-Z producers using the solid phase-amplified radioimmunassay procedure and poly [d(G-br^{5}C)] as a substrate.

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ON THE POSSIBLE ROLE OF SODIUM PERMEABILITY OF CELL MEMBRANE IN MITOGENESIS: A CRITICAL EVALUATION OF SOME METHODICAL APPROACHES

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SUMMARY

Experimental observations are reviewed supporting the unified theory on the basic mechanism of normal mitotic control and oncogenesis (Cone, 1971). Special attention is paid to the results regarding the intracellular ionic composition of various in vitro and in vivo systems by means of X-ray microanalysis and atomic absorption spectrophotometry. Contradictory conclusions drawn from the atomic absorption spectrophotometric measurements are explained by the methodical pitfalls of the preparative method: it is shown that the washing of cell cultures with Na^+-free media removed not only the rests of the extracellular ions but also a very great portion (above 90 %) of the intracellular Na^+ content. At last some of the possible mechanisms of regulation of mitogenesis by the membrane potential are listed and discussed in light of some recently published observations. Main conclusion of the authors is that the membrane hypothesis of mitogenesis, although cannot be regarded yet as a definitively explored mechanism of mitotic control, deserves great interest and further experimental testing.

INTRODUCTION

It is well known that the plasma membrane of the living cells maintains a strictly regulated asymmetric ion distribution between the extra- and intracellular space. In resting, differentiated cells the intracellular K^+ content is very high, whereas the Na^+ content is low as compared to the extracellular ion composition. This asymmetric distribution is due partly to the difference in the passive permeability of the membrane for these ions: it is well established that the ratio $P_K:P_{Na}$ in resting cells amounts to about 30 or even more (Adelman, 1971; Hope,

1971). Nevertheless, the cells must also sacrifice a very considerable portion of their energy yield (30-50 %, according to various estimations), in order to assure this asymmetric ion distribution. As a result of these efforts of the living cells, a so-called resting potential (RP) is built up on the cell membrane: the intracellular side is more negative than the extracellular one by about -40 to -100 mV. The significance of this RP is well known from the point of view of cell excitability. Namely, if the membrane permeability ratio $P_K:P_{Na}$ decreases under the effect of chemical transmitters like acetylcholine, etc., the asymmetric distribution tends to disappear, i.e., Na^+ enters and K^+ leaves the cell, meanwhile, the RP changes toward 0 or even to positive values. In a usual interneuronal or neuromuscular junction such a depolarization of the membrane takes place only for several mseconds, since the physiological system eliminates the presence of the chemical transmitter very quickly and pumps out also the excessive Na^+ from the cell, reexchanging this Na^+ for extracellular K^+ by using again energy.

The excited state of the cells manifests itself e.g., in contraction of the muscle cells, or in increased (or even decreased) secretion of the glands, etc. Although actually nothing is known about the biochemical mechanisms transforming the short-term depolarizations of the nerve cell membranes into relevant informations fixed reversibly in the memory of the brain, etc., it is evident that the short membrane depolarization must have some well-defined biochemical influence on all type of cells.

The electrophysiological knowledge suggests automatically another question: What can be the effect of the membrane depolarization for the cellular status, if it is maintained for much longer time, e.g. for minutes or hrs? This question was answered by Cone (1971): he proposed that sustained depolarization of the cell membrane may be of mitogenic effect both in normal and tumor cells. This assumption will be called membrane hypothesis of mitogenesis (MHM). Although this idea was attractive, it has gained only little attention for a relatively long time, until only circumstantial evidence had been available. The

situation was changed considerably by the observation showing that a specific Na^+-channel blocking drug (amiloride) can inhibit the cell proliferation in the regenerating liver in vivo and also in hepatocyte cultures in vitro (Leffert and Koch, 1979a, 1979b; Koch and Leffert, 1979). Since that time an extensive activity has been realized in this field, and the present work is intended (i). to summarize briefly the actual situation; (ii). to point out that some of the obviously contradicting results derive from methodical pitfalls; (iii). to list some of the possible mechanisms underlying the regulatory role of the membrane potential in the mitogenesis.

A SURVEY OF THE MOST RELEVANT EXPERIMENTAL RESULTS

1. Indirect approaches

The basic experiments were carried out by Cone (1971) on diploid Chinese hamster ovary cells. The model was the following: Naturally synchronized cell cultures were kept in various media simulating the naturally occurring range of the RP from -10 to -90 mV. DNA synthesis and changes in the cell number were followed in such experiments. It has been established that the cells are growing until the RP is higher than -45 mV, a complete arrest of mitoses was observed at -75 mV, and the block of DNA synthesis and mitosis was completely reversible (Cone, 1971).

Similar experiments were carried out by Orr et al. (1972). They used baby-hamster kidney cells and varied the extracellular ionic composition by increasing the K^+ content by equimolar replacement of Na^+. They observed a biphasic effect of extracellular potassium, namely, 72 mM extracellular K^+ (causing a depolarization from about -57 mV to approximately -19 mV) increased the cell growth, whereas 114 mM K^+ or more in the extracellular space inhibited it. (This latter case represents an RP of -8.9 V.) Since the eventual limit of depolarization which is still stimulating has not further been explored, one cannot decide actually, whether the observation of Orr et al. (1972) is specific only for the kidney cells or it should be

attributed to the fact that the excessive replacement of the extracellular Na^+ with K^+ creates really unphysiological circumstances blocking the growth of the cell culture.

The contact inhibition was studied in 3T3 and Chinese hamster ovary cells (Cone and Tongier, 1973). It has been shown that a 5-6-fold increase in the RP is associated with the mitotic arrest at saturation cell densities. When RP was high, the intracellular Na^+-content was low, and vice versa.

DNA synthesis and mitosis have been induced in fully differentiated neuroblast cultures by depolarizing the membrane of these cells for long periods with a variety of drugs like ouabain, veratridine and gramicidine (Cone and Cone, 1976). All these drugs have the same effect: they increase the intracellular Na^+-concentration and decrease the potassium content, i.e. depolarize the membrane. The result of such treatments was an induction of DNA synthesis in 31-63 % and the appearance of two nuclei in 20-30 % of the cells (Cone and Cone, 1976).

Apart from some further data giving support to the MHM (Borgens et al., 1977; Rodan et al., 1978; Smith and Rozengurt, 1978, etc.), one can find even some negative conclusions: Sachs et al. (1974) were of the opinion that, although the membrane potential of isolated cells of Chinese hamster lung fluctuates during the cell cycle, the intracellular sodium content plays no causal role as a regulator of mitotic activity.

2. Measurement of the intracellular ionic composition in various models

Sustained depolarization of the cell membrane can manifest itself under physiological conditions in the increase of the intracellular Na^+-content for a considerably longer time (minutes or hrs) than during the normal excitation of the cells. Obviously, in such cases one can measure an increase of the intracellular Na^+-concentration and also of the Na^+/K^+ ratio. True intracellular concentrations of Na^+ and K^+ can be measured by X-ray microanalysis and atomic absorption spectrophotometry, if the specimen preparation technique can preserve these ions on their place, i.e., within the cells. We shall give a brief survey of the typical results obtained by using these techniques.

a.) X-ray microanalysis

Various methods applying energy-dispersive X-ray microanalysis have been used to determine the intracellular Na^+ and K^+ concentrations in cellular models both in vivo and in vitro. The so-called bulk-specimen method has recently been critically reviewed (Zs.-Nagy, 1983). Data have been published on: (a) transformed cells (hepatomas and mammary adenocarcinomas); (b) normal counterparts of the transformed cells; (c) normal, rapidly dividing cells; (d) normal, slowly dividing cells (Cameron and Smith, 1980; Cameron et al., 1980; Smith et al., 1978); (e) invasively growing human cancers (Zs.-Nagy et al., 1981, 1983; Lukács et al., 1982). All these results supported the existence of a correlation between the high sodium content (and the high Na^+/K^+ ratio) and the proliferative capacity (clinical malignancy) of the cells. Although these data still do not prove the causal role of membrane depolarization (i.e., high intracellular sodium content) in the regulation of cell proliferation, they gain a certain significance in the light of some further experimental observations obtained with amiloride (see below).

b.) Atomic absorption spectrophotometry

This method of determination of the intracellular cation concentrations has been applied in cultured cells, like BALB/c-3T3 cells (Frantz et al., 1981; Sanui and Rubin, 1982) chick embryo fibroblasts (Moscatelli et al., 1979; Sanui and Rubin, 1977, 1978, 1979). A common feature of the method used by these authors is that the cell cultures are washed with sodium-free media several times before the determination of the ionic content takes place, in order to eliminate the contribution of the extracellular ions to the measured value. However, the results obtained by these authors are rather contradictory even to each other: the figures for Na^+-content may vary by a factor of 5 to 15 even under similar experimental arrangements (e.g., Frantz et al., 1981; Sanui and Rubin, 1979). On the basis of the data measured with this technique, no clear correlation was detected between the proliferative capacity and the intracellular Na^+-concentrations of the cells, therefore, these

authors categorically deny the regulatory role of the intracellular Na^+ (i.e., the sustained depolarization) in mitogenesis (Moscatelli et al., 1979; Sanui and Rubin, 1977, 1978, 1979, 1982). We intend to point later to the methodical pitfall of the preparative technique used by these authors for the preservation of the intracellular Na^+ content on its place.

3./ The effects of Amiloride

Amiloride (Glitzer and Steelman, 1966) is a potassium sparing diuretic with the chemical formula of 3,5-diamino-N-(aminoiminomethyl)-6-chlorpyrazine-carboxamide-HCl. Its primary site of action is in the distal nephron, where it interferes with the exchange of sodium and potassium (Baba et al., 1968), since it is a specific Na^+-influx inhibitor (Salako and Smith, 1970). Further details regarding its pharmacology can be found in the review of Macfie et al. (1981).

The basic observation was made by Epel (1977): amiloride was found to block the initiation of DNA synthesis in fertilized egg cells. Starting from this observation, it has been demonstrated that sodium influx is necessary to initiate rat hepatocyte proliferation (Koch and Leffert, 1979; Leffert and Koch, 1979a, 1979b), since amiloride could block the Na^+-influx and also the proliferation of hepatocytes in vivo after partial hepatectomy and in vitro in cultures.

The inhibitory effect of amiloride on the cell proliferation has been confirmed in various cellular systems: neuroblastoma cultures (Moolenaar et al., 1981; Mummery et al., 1982); fibroblasts (Villereal, 1981, 1982); L-1210 murine leukemia cells (Pieri et al., 1982); hepatocytes and hepatoma cells (Pieri et al., 1983).

Although the question of mitotic regulation by the monovalent cation transport cannot be considered as a definitively resolved problem (see for details: Boonstra et al., 1982), one has to analyze very carefully all the observations in this field, since we badly need a useful explanation for the events of the cell division both in normal and cancer cells.

CRITICAL EVALUATION OF THE PREPARATIVE METHOD FOR MEASURING INTRACELLULAR CATION CONTENTS IN CULTURED CELLS BY ATOMIC ABSORPTION SPECTROSCOPY

As it has been mentioned in the previous parts of this paper, atomic absorption spectroscopy on cultured cells revealed very contradictory results regarding the intracellular Na^+ content during various phases of the cell cycle (Moscatelli et al., 1979; Sanui and Rubin, 1977, 1978, 1979, 1982). These authors determine the "intracellular"cation contents of the cells being attached to the plastic Petri dishes after several washings (5 to 7 times) with Na^+-free sucrose solution. The cells remaining on the wall of the dish are collected mechanically after washings and protein as well as cation contents are measured. The results are expressed in mole cation/protein weight units. We intend to show that the results obtained this way are influenced by a considerable error of preparation, therefore, the conclusions based on them cannot be accepted.

1./ The values of cation concentrations expressed on protein basis mentioned above can be converted into more usual units like mmole cation/liter of cell volume. Table 1 reports the converted data taken from Fig. 1 of Sanui and Rubin (1979), assuming that the BALB/c-3T3 fibroblasts contain 80 mg protein per 1 g fresh weight. We draw 2 conclusions from the data of Table 1. First, it is evident that the omission of any washing results in irrealistically high "intracellular" cation contents, due especially to the very high Na^+-concentration. This can be explained by the rather high sodium content of the culture medium (150 mM), and its apparently incomplete removal from the dishes by the first aspiration. Secondly, it is also evident from the data of Table 1 that already after the first washing there is a very dramatic decrease of the "intracellular" cation contents, resulting in an irrealistically low total cation content (80 mmole per liter cell volume even together with the corresponding anions represents only about 176 mequ/l ionic strength, if we consider the proportion of divalents). Furthermore, it is noteworthy that the sodium content of the

Table 1.

Protein content and calculated intracellular cation concentrations of cultured fibroblasts from Fig. 1 of Sanui and Rubin (1979).

Number of washings	Protein mg/dish	Na^+	K^+	Mg^{2+}	Ca^{2+}	Total cations
0	19.2	760.0	76.0	8.0	8.0	860.0
1	12.0	20.0	56.0	4.8	1.2	80.0
2	12.0	4.0	56.0	4.8	1.0	68.0
7	12.0	2.6	56.0	4.8	1.0	64.0

Note: Cation concentrations are given in mmole/liter cell volume, assuming 80 mg protein per g fresh weight. Total cations are calculated from the corresponding curve of the cited authors: the obtained figures are not equal with the sum of the single cations due to the error of reading the values from the graphical plots (valid also for Table 2).

Zero washing means cultures whose culture medium was removed only by aspiration.

fibroblasts decreases during the further washings to very low values: 2.6 mmole Na^+ per liter cell volume cannot be considered as a physiological value. Other authors applying quick rinsing instead of the relatively longer washings, obtained 40 mmole Na^+ per liter of cell volume in dividing fibroblasts and 15 mmole/l in the density-arrested ones (Frantz et al., 1981). It is very important that protein content and the other cations (K^+, Mg^{2+} and Ca^{2+}) do not decrease any more after the first washing, whereas the Na^+ does. In another paper Sanui and Rubin (1978) have demonstrated that after 5 usual washings, the sodium content of cells still decreased about 8-10 %, if a sixth sucrose washing of 10 seconds was applied.

2./ Sanui and Rubin (1979) calculated whether the repeated washing with sucrose can completely remove the culture medium from the cells (see the cited paper, pp. 218-219). Of course, they concluded that the washing method was perfect for this purpose. Nevertheless, no calculation was made for checking whether the washings can remove cations from the cells. We are going to show in Table 2 that e.g. the data taken again from Fig. 1 of Sanui and Rubin (1979) indicate such a removal of the true intracellular cation content. The basis of such a calculation is the following:

The total amount of the culture medium used for one measurement is known (87.5 ml), and the cation content is also indicated by Sanui and Rubin (1979): Na^+ = 150.0 mM, K^+ = 5 mM, etc. It is also described in the cited paper that the Dulbecco's Modified Eagle's medium used for the BALB/c-3T3 fibroblasts was completed with 10 % calf serum. Unfortunately, Sanui and Rubin (1979) did not specify the type of the calf serum used, therefore, we had to assume that it was a dialyzed type which is of must common use, and its protein content was 6.5 g% (Adams, 1980). It means that the culture medium contained 0.65 g% protein, i.e., the cation concentrations of the medium can be calculated on a protein weight basis, e.g., in µmole cation/mg protein units (Table 2).

It is evident from Fig. 1 of Sanui and Rubin (1979) that the protein content of the fibroblast fraction decreased only during the first washing and remained constant thereafter

Table 2

Comparison of de facto and expected protein and cation losses during the first sucrose washing of cultured fibroblasts (from the data of Fig. 1 of Sanui and Rubin, 1979).

Experiment or parameter	Protein mg/dish	Na^+		K^+		Mg^{2+}		Ca^{2+}		Total cations	
		A	B	A	B	A	B	A	B	A	B
No washing	19.2	9.5	182.4	0.95	18.24	0.1	1.92	0.1	1.92	10.7	205.4
After 1st wash.	12.0	0.25	3.0	0.70	8.4	0.06	0.72	0.015	0.18	1.0	12.0
De facto removed quantities	7.2	24.92	179.4	1.37	9.84	0.17	1.20	0.242	1.74	26.86	193.4
Expected removal		23.07	166.1	0.77	5.54	0.12	0.86	0.262	1.89	24.23	174.5
De facto removed in % of exp. removal		108		178		140		92		111	

Notes: A = µmole cation/mg protein; B = total amounts in µmoles/dish

Although the de facto removed Na^+ by the first washing is only 108 % of the expected value, this difference represents approximately 82 % of the true intracellular Na^+. Further washings remove another 15 % of the cell Na^+-content so that after 7 washings only about 2.5 % remains in the intracellular space.

(Table 1). It seems to be quite safe to assume that the amount of proteins washed out by the first washing represents the extracellular proteins present in the rests of the medium remaining still in the Petri dish after the aspiration of the liquid phase.

If the washings have no influence on the true intracellular cation contents, the values of µmole removed cation per mg removed protein should be equal (within the experimental error) with the values calculated from the composition of the culture medium. This latter figure is called "Expected removal" in Table 2. In other words, the cation contents of the dishes should decrease during washings in a rather strict proportion with the removal of the extracellular proteins. Table 2 demonstrates that this expectation is fulfilled in none of the cases of the 4 cations studied. Na^+, K^+ and Mg^{2+} were removed to a higher extent, Ca^{2+} to a lower one than one can expect during the first washing on the basis of the removed protein amount. Moreover, the Na^+-content of the fibroblasts decreased very considerably up to the 5th washing (to 0.33 µmole/mg protein), meanwhile no further amounts of protein was removed during the 2nd and further washings (Table 1). This fact indicates that this kind of washing procedure seriously reduces the intracellular Na^+-content of the fibroblasts: less than 3 % of Na^+ remains in the cells after the 7th washing.

It should be noted that the eventual metabolization of the proteins of the culture medium cannot contribute considerably to these results. Namely, even if the total amount (12.0 mg) of cellular proteins found after the first washing derives from the serum-protein content of the medium, it represents only about 2 % of the total proteins (568.75 mg) in the 87.5 ml volume of the culture medium.

The behaviour of intracellular Na^+ during the washings is logical. It is well-known, that the intracellular Na^+-content is regulated mainly by the Na^+-K^+-dependent-ATP-ase, if the Na^+-permeability of the cell membrane is in the physiological range. This enzyme working usually against the high extracellular sodium gradient, will be much more efficient when the extracellular sodium concentration decreases drastically, and will quickly pump out the intracellular sodium until a quite new and non-physiological equilibrium will be reached.

We are of the opinion that the results obtained with the method of Sanui and Rubin (1979) in cultured cells for the intracellular cation contents, especially for Na^+, cannot be regarded as true intracellular concentrations. It should be stressed that the best method actually available for the immobilization of the intracellular light elements like Na^+ is the quick deep-freezing as applied when preparing specimens for X-ray microanalysis (Zs.-Nagy, 1983), since any kind of intervention at room temperature like rinsing and washing in and with Na^+-free media displaces a considerable portion of the Na^+-content from the intracellular space.

The conclusion can be drawn from these considerations that the MHM cannot be refuted on the basis of data obtained from measurements performed on cultured cells by atomic absorption methods applied after washing the cells. It is also evident that the omission of the washings gives also very great errors (Table 1), i.e., this kind of specimen preparation is unsuitable to preserve the real intracellular concentration of Na^+.

ON THE MECHANISM OF REGULATION OF MITOGENESIS BY THE MEMBRANE POTENTIAL

Although the amiloride-experiments mentioned above offer a quite convincing proof for the causal role of Na^+-influx in the initiation of cell proliferation, there are several questions to be answered before considering the MHM as the basic mechanism of mitotic regulation. The increased intracellular Na^+-content in itself may have some direct effect on the nucleic acid-protein complexes. Namely, ions can produce some nonspecific electrostatic interactions with macromolecules, and they can alter the conformation and solubility of macromolecules by specific effects (Hippel and Schleich, 1969). The charging properties, the conformation and solubility of macromolecules are directly related to the functions of living systems. Therefore, one can assume that the effects of ions

are of great importance, in spite of the fact that such effects on the transition temperature for a polymer chain aggregation process are still not clearly understood (Hey et al., 1976). The so-called polyelectrolyte theory (Douzou and Maurel, 1977a, 1977b; Maurel and Douzou, 1976) offers a wider basis of explanation for the ionic effect on the nucleic acid-enzyme complexes (see for more details: Zs.-Nagy, 1979).

There is another possibility for the mechanism of action. Namely, one can suppose the existence of directly potential dependent proteins involved in the mitotic regulation. The reality of such an assumption is supported by the fact that some proteins respond to the depolarization of the brain cell membrane by reversible phosphorylation (Forn and Greengard, 1978) independently of the method of depolarization (10-fold increase of the extracellular potassium or veratridine adminstration).

The mechanism of the increase in intracellular Na^+ content (i.e., of the depolarization of the cell membrane) in vivo is unknown. Theoretically, one can assume two basic processes, either of which (or their combination) may result in this increase. First, it is possible that the Na^+-permeability of the cell membrane remains normal, but the membrane-bound Na^+-K^+-dependent ATP-ase (the "pump enzyme") is defective, thereby allowing more or less quickly the extra- and intracellular space to equilibrate the Na^+-gradient. Secondly, it is possible that the Na^+-permeability of the resting cells increases above the usual, normal level. As a consequence of increased Na^+-influx, the pump-enzyme will be activated to the uppermost limits but even such a high level of pumping may not be sufficient to maintain the physiological Na^+-gradient. It is interesting that both of the above mentioned mechanisms may be associated with cancer growth. E.g. in chemically induced liver cancers, the disappearance of the pump-enzyme was shown to be the very first and persisting detectable histochemical alteration (see for details: Emmelot and Scherer, 1980). On the other hand, histochemical and biochemical studies performed on the papillary cancers of human thyroid gland revealed a very considerable (5 to 10-fold) increase of the pump-enzyme activity as compared

to the normal thyroid epithelial cells (Mizukami et al., 1983). Although this fact still does not directly prove an increased sodium permeability of the cancer cell membrane, it offers a strong support for such an assumption. Similarly, an increased sodium permeability of serum-stimulated fibroblasts against the quiescent cells, or of transformed cells against normal cells has been demonstrated (Rozengurt and Mendoza, 1980). The possibility that human cancerogenesis may result from an increased sodium permeability of the cell membrane may also be of great practical importance. It seems to be possible to produce some specific sodium channel blocking drugs capable of inhibiting cell proliferation in vivo like amiloride does in experimental systems (Leffert and Koch, 1979a, 1979b; Koch and Leffert, 1979).

The last question of this discussion is the role of calcium in the mitogenic regulation. It is well established that this divalent cation is an important inductor of cell proliferation in numerous systems (Epel, 1980; Jaffe, 1980; Whitfield et al., 1980). There is no doubt that these observations are correct. The role of calcium gains an important interpretation in the light of the results published recently (Villereal, 1981, 1982; Owen and Villereal, 1982): it has been demonstrated that most of the calcium effects on the mitosis are amiloride-sensitive, i.e., the target of calcium may well be the sodium channel protein.

In July 1982 a special symposium was organized at Lake Placid, N.Y. under the title "Ions, Cell Proliferation and Cancer". Numerous aspects of the mitotic regulation have been discussed there, among others the possibility that the high Na^+-influx may result in a rise of intracellular pH (about 0.1 to 0.2 units) and this pH shift may be sufficient to provoke the initiation of cell division. Materials of that Symposium are being published according to our best knowledge.

We are convinced that although the MHM cannot yet be regarded as a definitively explored and accepted mechanism of mitotic regulation, it deserves great interest and open-minded theoretical considerations in the future.

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BHARGAVA:

Did you ensure that in your measurements of Na^+ using the electron microscopic X-ray dispersive analysis technique, you did not also score a nucleus lying underneath the one you were looking at?

As I have pointed out earlier, one must distinguish between the crucial and central event (the "switch") more or less directly controlled by the mitogen, and the events of the programme of the division cycle. For example, the ratio of dATP to ATP would be much higher in dividing cells than in resting cells, and so would be the case with many other cellular constituents. How would these cases be different from the case of Na^+ you mention? Surely, they all cannot be the switch!

The mitogen does not directly control the S-phase, which is controlled by the events (such as the synthesis of certain specific proteins) in the G_1 phase.

ZS.-NAGY:

The method we apply for measuring the Na^+-content of cell nuclei is well defined as regards the penetration depth of the electron beam and all other circumstances. You can find all these data in my recent review (Zs.-Nagy, 1983). There is no risk to overpenetrate to the adjacent cell, when measuring cancer cell nuclei.

The problem of switch of mitosis is of course the most important question. Of course, many other parameters can be changed in correlation with the mitogenesis, and they all cannot play the role of switch. We assume that the sustained increase of the Na^+/K^+ ratio, i.e., the depolarization of the membrane to a certain extent for a longer time than during the usual excitation of the cell, may represent the primary switch, since some of the cellular components are extremely sensitive to such changes. The experiments with Amiloride are actually the most important

arguments in favour of this assumption, nevertheless, the situation is far from being completely clear.

I agree with you. The mitogens act already, if they are bound to the cell membrane, and their very first action is an alteration of the Na^+ and K^+ permeability of the membrane. The action of mitogens can be interpreted in terms of the MHM, although this possibility has not been recognized widely enough.

MARTONOSI:

Of course, developmental controls are complicated yet we are naturally attracted by simple models since we cannot tell in advance which of these models contain an element of truth and which are misleading, it is wise to consider all of them seriously.

Changes in cytoplasmic Na^+ are likely to play some regulating role in cell proliferation but I view these changes only as parts of an amplification process leading to a set of signals that directly affect metabolic pathways. The reason for this view is that the described changes in a cytoplasmic Na^+ are relatively minor at most 2-3-fold which are insufficient to explain several hundredfold changes in processes required for cell proliferation such as for example DNA synthesis or the synthesis of certain proteins.

On the other hand Ca^{2+} concentrations may undergo changes of several orders of magnitude in the cytoplasm that are suitable signals to affect metabolism directly.

Therefore it is likely that changes in cytoplasmic (Na^+) are translated via Na^+:Ca^{2+} exchange systems into changes in (Ca^{2+}) and that Ca^{2+} or some Ca^{2+} induced responses further up the amplification cascade trigger cell proliferation.

At this time it is impossible to tell whether any particular event is permissive or causative for cell proliferation or frequently whether it is even related to it.

ZS.-NAGY:

I want to comment first the role of Ca^{2+} in the mitotic regulation. As I pointed out in my lecture, many of the Ca^{2+}-effects proved to be amiloride-sensitive, i.e., they are realized by influencing the Na^{+}-permeability of the cell membrane. Therefore, I agree with the logic of Cone (1971) in the interpretation of the cell behaviour: If you consider the possibilities of the evolution, you have to admit that the primitive forms of living material evoluted in the presence of very high sodium concentration. We can speculate that most probably this high sodium content was an advantage at first, since it allowed a high rate of macromolecular reproduction, however, later on it became an inhibitory factor of differentiation. Therefore, the appearance of the Na^{+}-pump enzyme represented a great selective advantage because it offered a possibility for the primitive living system to become free from the external Na^{+}-concentrations. This could be one of the absolute conditions of differentiation, however, the cellular system preserved its high sensitivity toward the high sodium-content (i.e., toward the depolarization). It cannot be accidental that all living systems sacrifice a very great percentage of their energy yield for the maintenance of the low intracellular Na^{+}. The Ca^{2+}-regulation of mitogenesis was probably built up on the sodium permeability as I mentioned in my lecture, i.e., it may be a very important fine regulatory factor, however, it cannot work, if we close the sodium channels by amiloride.

There is one point in which I disagree with you. I do not believe that the causative or permissive character of a regulatory mechanism can be judged on the basis of how large percentual changes can be recorded for one or the other parameter, as you suggested. 2-3-fold increases of the intracellular Na^{+}-content may be much more important and efficient in triggering mitotic activity, if the Na^{+}-content (i.e., the depolarization of the membrane) is really relevant to the initiation of DNA synthesis, than several hundred-fold increases of any other parameter being not relevant from this point of view.

RESCH:

Events which can be measured in cells during the mitotic activation can fall principally into three categories:
a.) They may belong to the chain of events linking the triggering event with DNA replication and mitosis, and thus be causative;
b.) They may be permissive, i.e., required for some metabolic processes to occur which are associated with growth and proliferation.
c.) They may be epiphenomena, or accidental events.
Unless you have no good evidence that any measured event belongs to the first category, - and a correlation between certain events and cell growth is definitely not sufficient - I like to assume that such changes belong rather to permissive or accidental events.
At the present stage it appears that changes in surface potential or amino acid transport rather belong to this category.

ZS.-NAGY:

Your classification is nice and I agree with it. However, the available experimental evidence, especially the effect of amiloride convinced us that a sustained increase of the Na^+/K^+ ratio, i.e., the sustained depolarization of the cells cannot be regarded any more as purely accidental events of the mitotic process. You can disagree with such an interpretation but you cannot disregard the well established experimental facts. The problem of mitotic regulation is far more serious than any other biological question, therefore, opinions or declarations cannot resolve anything. We stressed in our lecture that extensive further experimentation is needed to learn more about the mechanism of this type of regulation, if it is really relevant to the initiation of mitosis. The main intention of our lecture was just to call attention to this approach, to point out to the controversary data, and give some perspective for further experiments. The MHM is an attractive working hypothesis for the clarification of which

we are ready to make further efforts. The future will show whether we are right or not.

WOJTCZAK:

For cell suspension there is a simple and reliable method of determining cell components: this is silicone-oil filtration.

ZS.-NAGY:

According to my best knowledge this method has not been used for checking the correctness of the membrane hypothesis of mitogenesis.

SOMOGYI:

If your explanation is correct in the case of subintoxicated patients by digitalis, where the intracellular Na is elevated, an increased mitosis rate should be observed. Have you any data about such an observations?

ZS.-NAGY:

I did not specifically collect data regarding the topics you are mentioning. Nevertheless, on the basis of the results obtained with ouabain cited in my lecture, I would not be surprised, if high doses of digitalis could increase the DNA synthesis and mitotic index in certain human tissues.

TIGYI:

I congratulate on your brilliant experimental results but I'd like to warn you in making definitive conclusions. There are old findings which have shown that many kind of different treatments on membrane (ultrasound, ionizing radiation, direct electric current) cause uniformly a Na^+ increase and K^+ decrease in the cell.

ZS.-NAGY:

As I emphasized in my lecture, we do believe that we have a promising working hypothesis in our hands, and no defini-

tive conclusions can be drawn for the time being. Nevertheless, I think that the data mentioned by you rather support than refuse the membrane hypothesis of mitogenesis in a general sense.

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PROTEINASE ACTION

Edited by

P. ELŐDI

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This volume contains the material of the international conference held in August 1983 in Debrecen.

The first part discusses the mechanism of proteolytic enzymes with special regard to the plasmin action and its regulation. In the second part the regulating role of these enzymes as well as the intracellular proteinases are dealt with. The next section examines the significance of the natural and artificial substrate of small molecule inhibiting proteinase action, while the fourth part surveys the isolation and mechanism of the proteolytic enzymes of plant and microbial origin.

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